INVESTIGATING ACUTE *MONTIPORA* WHITE SYNDROME IN KĀNE‘OHE BAY, O‘AHU: CAUSATIVE AGENTS, PUTATIVE ENVIRONMENTAL DRIVERS, AND THE IMPORTANCE OF HOST HEALTH

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ABSTRACT

Reports of disease-related coral mortality have increased over the last few decades. Coral diseases contribute to the decline of coral reefs globally and threaten the health and future of coral reef communities. There is an imminent need to develop our understanding of the biotic and abiotic drivers of coral disease outbreaks on an ecological and molecular level. *Montipora* white syndrome (MWS) is a tissue loss disease that affects populations of the coral *Montipora capitata* in Kāneʻohe Bay, Hawaiʻi. Two types of MWS have been documented; a chronic progressive tissue loss disease termed chronic MWS (cMWS**)**, and a comparatively faster infection termed acute MWS (aMWS). Colonies exhibiting cMWS have been observed to spontaneously switch to aMWS in the field. This dissertation describes analysis of coral-associated bacterial communities, causative agents of disease, the importance of host health, and putative environmental drivers that may be promoting outbreaks of aMWS and the switching of chronic infections to acute lesions. This investigation of aMWS is described over four chapters. First, the bacterial communities between healthy and diseased *M. capitata* colonies were compared during an ongoing aMWS outbreak versus a non-outbreak period to identify whether a specific shift in bacterial community structure is associated with this disease. The bacterial communities were analyzed using high-throughput sequencing and all health states shared different community compositions with an overall high abundance of *Escherichia* spp. possibly originating from sewage contamination. Second, a coral disease treatment method was assessed to determine whether the removal of cMWS lesions from *M. capitata* colonies could reduce morbidity and prevent re-infections. The treatment resulted in an overall

reduction in morbidity and prevented lesions from switching from chronic to acute tissue loss. Third, to further describe potential causative agents of this disease, bacterial isolates from diseased *M. capitata* were screened for virulence using controlled infection experiments. Isolate OCN003 was identified as an etiological agent of aMWS, and more readily infected cMWS-afflicted coral fragments than healthy fragments, which is the first coral pathogen demonstrated to act as a secondary pathogen. Lastly, OCN003 genome was sequenced and was identified as a novel bacterial species and named *Pseudoalteromonas piratica.*

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CHAPTER 1: INTRODUCTION TO CORAL DISEASE AND CORAL PATHOGENS

The coral holobiont

Reef-building corals thrive in the oligotrophic waters of the tropics and are a multi-organismal association comprised of the coral animal, endosymbiotic algae, and a suite of microbial organisms, such as bacteria (Rohwer *et al.*, 2001, 2002; Bourne and Munn, 2005), archaea (Kellogg, 2004; Wegley *et al.*, 2004; Siboni *et al.*, 2008), viruses (Patten *et al.*, 2008), fungi (Oppen *et al.*, 2009), and protozoa (Aladro-Lubel and Martínez-Murillo, 1999; Rohwer and Kelley, 2004). The microbes living in symbiosis with the coral host inhabit various coral niches, including the coral calcium carbonate skeleton, the tissue layers, and the surface mucus layer (Rosenberg *et al.*, 2007). The extracellular mucus layer of corals is considered to be the first line of defense against foreign invaders, such as pathogenic microorganisms (Ritchie, 2006; Sharon and Rosenberg, 2008; Garren and Azam, 2012). Mucin, containing different sugars, lipids, and proteins, is produced by coral mucocytes and makes up the extracellular mucus layer. The constant release of mucin, along with ciliary action, allows for the removal of sediment, debris, and microorganisms from the coral surface (Ducklow and Mitchell, 1979; Garren and Azam, 2012). Coral-associated bacteria are believed to contribute to coral nutrition through the biogeochemical cycling of nitrogen, carbon, and sulfur compounds and contribute to coral health by acting as the first line of defense against pathogen invasion of host tissues (Shnit-Orland and Kushmaro, 2009; Kimes *et al.*, 2010). Coral-associated bacteria form a physical barrier against

pathogen attachment and colonization and are hypothesized to produce antimicrobial compounds that prevent the proliferation of potentially pathogenic bacteria (Ritchie, 2006; Rypien *et al.*, 2010).

Worldwide coral reef health decline

Coral reefs are the most diverse and productive ecosystems on earth, and millions of people rely on them as their major source of food and income (Moberg and Folke, 1999; Worm *et al.*, 2006). Local economies depend on the revenue resulting from tourism and recreation, and research suggests that coral reefs harbor an unknown number of organisms that produce chemical compounds beneficial in the treatment of human diseases (Cragg *et al.*, 1997). Despite their significance and beauty, coral reefs existence is threatened by the impacts of anthropogenic stressors, such as climate change, pollution, habitat loss, and overfishing (Richmond, 1993; Harborne *et al.*, 2017). Human activities have led, and continue to lead, to a global decline in coral reef health and coral coverage (Hughes, 2003; De'ath *et al.*, 2012). Some of these anthropogenic stressors have immediate short-term effects while others have more insidious long-term impacts on coral reefs. Smothering by sediment after a winter storm on Maui in 2002 led to a 33% coral cover reduction in Honolua Bay (Dollar and Grigg, 2004). Land reclamation and dredging in the United Arab Emirates resulted in reef ecosystems being removed and large areas of seabed being filled, which was an immediate reduction of coral coverage accompanied by the release of large volumes of suspended sediments into the coastal environment (Rezai *et al.*, 2004). Chemical pollutants and excess nutrients, such as sewage contamination, usually do not cause immediate damage to corals, and therefore, cannot be easily determined to be a

direct harm to corals. However, numerous sewage components have been shown to affect corals; freshwater lowers salinity which can lead to coral mortality, dissolved inorganic nutrients have been demonstrated to decrease coral fecundity and skeletal integrity, and steroidal estrogens have been shown to reduce the number of coral egg-sperm bundles and overall reduce coral growth rates (reviewed in Wear and Thurber, 2015).

The increase in greenhouse gasses predicted over the next decades will lead to elevated sea surface temperatures (SST) and ocean acidification, which are detrimental to the health and integrity of corals (Hoegh-Guldberg *et al.*, 2007). Increased SST can lead to coral bleaching, which causes the loss of the symbiotic algae within the coral tissue (Hoegh-Guldberg, 1999). These mutualistic dinoflagellates (*Symbiodinium* spp.) provide the coral animal with fixed carbon, thus, the loss in energy production that occurs during bleaching is immense (Anthony *et al.*, 2008; Roth, 2014). A "bleached" coral relies solely on heterotrophic feeding, a state that will slowly starve a coral. It is therefore likely that prolonged elevated SST will eventually lead to complete coral mortality and extensive loss of coral cover worldwide (Anthony, 2016).

Another phenomenon that has been detrimental to reefs are coral diseases. Reports of different coral diseases have been increasing around the world over the last 30 years (reviewed in Woodley *et al.*, 2016). During this time, these diseases have increased both in frequency and severity and caused major community shifts to macroalgal dominance on Caribbean reefs (Aronson and Precht, 2001). Our knowledge regarding coral diseases and their causative agents remains cursory and only a few research groups are investigating this interdisciplinary research topic. For many coral reef communities worldwide, no information regarding their health is available primarily due to limited resources. There is

an imminent need to fill these gaps and continue to develop our understanding of the biotic and abiotic drivers of coral disease outbreaks on an ecological and molecular level. Only then we will be able to design management strategies that can help save and conserve our coral reefs.

Previously described coral diseases and coral pathogens

Following the first report of coral disease in 1973 (Antonius, 1973), over 40 coral diseases from at least 75 countries have been described and characterized (reviewed in Woodley *et al.*, 2016). More than 200 species of reef-building corals are affected by different diseases, and reports of mass mortality events have been increasing over the last two decades (Harvell *et al.*, 1999; Jones *et al.*, 2004; Bourne, 2005; Brandt *et al.*, 2012; Aeby *et al.*, 2016). Despite the continuous emergence of new coral diseases, only a small number of etiological agents have been characterized. Koch's postulates of disease causation are a set of guidelines that are commonly used to infer the causality of a microorganism as a disease agent (Koch, 1876): (I) The microorganism must be present in all organisms suffering from the disease and should be absent in healthy organisms, (II) the microorganism should be isolated from a diseased organism and can be grown in pure culture, (III) the cultured microorganism should cause disease symptoms if inoculated into healthy hosts, and (IV) the same microorganism must be re-isolated from the inoculated, diseased hosts. These postulates have been utilized by researchers to describe causative agents of coral diseases, although few causal relationships linking pathogens to a coral disease have been successfully demonstrated to date (Ben-Haim and Rosenberg, 2002; Patterson *et al.*, 2002; Denner *et al.*, 2003; Sussman *et al.*, 2008; Ushijima *et al.*, 2012, 2014,

2016). Diseases with known etiologies are: Black band disease (BBD), White pox, Aspergillosis, White plague (WPD), *Porites* trematodiasis, Yellow band disease (YBD), bacterial Bleaching, and White Syndrome in *Acropora* and *Montipora* (Rosenberg *et al.*, 2007; Ushijima *et al.*, 2012, 2014, 2016; Aeby *et al.*, 2015).

Black band disease (BBD) was first described in Belize in 1973 (Antonius, 1973). It has since been found to affect various coral genera throughout the world (Bruckner *et al.*, 1997; Sutherland *et al.*, 2004). BBD is caused by a microbial consortium dominated by filamentous cyanobacteria, which is responsible for the characteristic black band (Richardson, 2004). Other constituents of the BBD lesion include sulfide-oxidizing bacteria (*Beggiatoa* spp.), sulfate-reducing bacteria (*Desulfovibrio* spp.), and various heterotrophic bacteria (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Barneah *et al.*, 2007; Aeby *et al.*, 2015). Although the consortium is diverse, pathogenesis is consistently due to concentrated sulfide and anoxic conditions underneath the BBD mat that kills the coral tissue (Richardson, 1996; Sutherland *et al.*, 2004; Rosenberg *et al.*, 2007; Richardson *et al.*, 2009).

White pox, also termed acroporid serratiosis (Patterson *et al.*, 2002) and patchy necrosis (Bruckner and Bruckner, 1997), was first documented in 1996 on reefs off Key West, Florida, and exclusively affects *Acropora palmata* (Holden, 1996). Since then, it has been observed on reefs throughout the Caribbean (Porter, 2001; Santavy *et al.*, 2001; Patterson *et al.*, 2002). White pox is characterized by irregularly shaped and sized areas of tissue loss throughout the colony. White pox is highly contagious and spread rapidly throughout the reefs in the Florida Keys National Marine Sanctuary during the mid 1990s killing over 70% of *A. palmata* colonies at certain reef locations (Porter, 2001). *Serratia*

marcescens was found to be the causative agent of this disease state (Patterson *et al.*, 2002). This is the first coral disease described to be caused by a fecal enteric bacterium, which indicates a possible association between White pox disease and sewage pollution.

The fungal disease, Aspergillosis, was first documented in 1995 and caused mass mortality of the sea fan, *Gorgonia ventalina,* in the Caribbean (Nagelkerken *et al.*, 1997; Kim *et al.*, 2006). Infections are characterized by purpling of the tissue that can lead to tissue loss. The causative agent has been identified as the fungus *Aspergillus sydowii* (Geiser *et al.*, 1998). *A. sydowii* is found in both terrestrial and aquatic systems, however, only the marine strains of *A. sydowii* have been shown to induce disease lesions (Geiser *et al.*, 1998; Sutherland *et al.*, 2004).

White plague disease (WPD) was first described in 1977 by Dustan. It is a rapid tissue loss disease responsible for numerous virulent outbreaks, reducing the overall coral cover on reefs worldwide (Richardson, 1998; Aronson and Precht, 2001; Richardson *et al.*, 2001; Navas-Camacho *et al.*, 2010; Pollock *et al.*, 2011). The Caribbean and the Indo-Pacific reefs have been most affected by this disease, which presents a pronounced line of bright, white tissue that separates the healthy tissue of the coral from exposed coral skeleton (Richardson *et al.*, 2001). This disease has been divided into three types: WPL I (Dustan, 1977), WPL II (Richardson *et al.*, 1998), and WPL III (Richardson *et al.*, 2001). Each type affects different coral species and displays increasing rates of tissue loss (Richardson *et al.*, 1998, 2001; Sutherland and Ritchie, 2004). The causative agent of WPL II in the Caribbean was proposed to be *Aurantimonas coralicida* (Denner *et al.*, 2003). Another bacterium, *Thalassomonas loyana,* was cultured from coral infected with a WPL-like disease in the Red Sea. However, neither of these two bacteria could be indisputably verified as the

responsible pathogen in successive studies. Therefore, it is uncertain whether a single pathogen or a bacterial consortium produces the WPD phenotype in different coral species.

Porites trematodiasis is caused by a digenetic trematode, *Podocotyloides stenometra* Pritchard (Cheng and Wong, 1974; Aeby, 1998). *Porites* trematodiasis affects *Porites* spp. throughout the Pacific (Cheng and Wong, 1974; Bray and Cribb, 1989). P*orites* spp. serve as the second intermediate host for the trematode and infection is characterized by the presence of pink, swollen nodules on the coral colony that persist until senescence or removal of the trematode by coral-feeding fish (Aeby, 1998). *Porites* colonies afflicted with this disease have been found throughout the Hawaiian Islands (Reed, 1971; Aeby, 1998) and have been reported from other areas of the Pacific (Bray and Cribb, 1989). Parasitized *Porites* colonies can experience a 50% reduction in growth that likely impairs their ability to compete for space and resources on the reef (Aeby, 1991).

Yellow band disease (YBD) is highly infectious and affects several scleractinian coral species of the Caribbean and Indo-Pacific. YBD manifests as blotches followed by a circular ring pattern with a pale yellow to white margin (Santavy *et al.*, 1999; Cervino *et al.*, 2001, 2004). Cervino *et al.* (2004) identified four distinct *Vibrio* spp., strains YB36, YBM23, YBFL3122 and YBFLG2A, which induced YBD in healthy *Montastraea annularis*. When inoculated in combination, the disease signs appeared more quickly and reflected the field observations more closely than when each isolate was inoculated separately (Cervino *et al.*, 2004). These *Vibrio* strains seem to target the symbiotic algae within the coral tissue rather than the coral animal. When the seawater temperatures increase, the symbiotic algae are lysed in the tissue of the coral host, which leads to compromised cell integrity, resulting in tissue lysis (Cervino *et al.*, 2004; Sutherland and Ritchie, 2004).

Pathogens targeting a symbiont of the coral host, as opposed to directly harming the coral animal, is not just a phenomenon in YBD. *Vibrio*-induced coral bleaching is also due to the disruption of the symbiotic relationship between the coral animal and the algae living within the coral tissue. Cervino *et al.* (2004) reported that *Vibrio* communities associated with coral increased in bleached corals. Bleaching of *Oculina patagonica* has been shown to be caused by *Vibrio shiloi* and remains one of the best described coral diseases to date. *V. shiloi* cells use positive chemotaxis to move toward the mucus of *O. patagonica,* adhere to the coral surface, and penetrate the coral tissue, where they secrete a proline-rich peptide that binds to the symbiotic algae (Banin *et al.*, 2001; Vattakaven *et al.*, 2006; Rosenberg *et al.*, 2007). This peptide inhibits photosynthesis conducted by the algae, which progresses to cell lysis (Banin *et al.*, 2001). Ben-Haim *et al.* (2003) described another *Vibrio*-induced bleaching process that is temperature-dependent involving *V. coralliilyticus* strain BAA-450 that is eliciting tissue lysis in the coral species *Pocillopora damicornis*. During infections at 24 °C, the intracellular dinoflagellates were degraded and the coral bleached. At 27 °C, infections resulted in tissue lysis and increased host mortality, and at temperatures below 23 °C, BAA-450 was incapable of infecting *P. damicornis*. During increased seawater temperatures (27-29°C), levels of extracellular protease activity is increased by BAA-450, which is proposed to lead to bacterial-induced tissue lysis (Ben-Haim *et al.*, 2003).

Like all animals, corals can be affected by diseases, especially if they are stressed. The dramatic increase of coral disease outbreaks worldwide is believed to be due to deteriorating water quality associated with anthropogenic pollutants and increased seawater temperatures. Together, these factors may contribute to the over-proliferation and colonization of opportunistic and pathogenic microbes in corals.

Coral disesae treatments

Morbidity- and mortality-reducing methods have been developed for many plant and animal species affected by disease (Nandakumar *et al.*, 2001; Warrell *et al.*, 2008), but only a few have been developed for corals. The removal of disease lesions is commonly used to cure some diseases affecting vertebrates and invertebrates. Lesion removal has also been established as an effective method to mitigate damage inflicted by coral diseases (Hudson, 2000; Dalton et al., 2010; Williams, 2013; Aeby et al., 2015). Studies have suggested corals to be efficient in healing damage done by re-growth of coral tissue over the affected area (Henry and Hart, 2005; Work and Aeby, 2010). Nevertheless, Aeby and Santavy (2006) demonstrated that injuries inflicted on *Montastrea faveolata* increased susceptibility to black band disease. Additionally, Page and Willis (2008) recently showed that injured coral was readily colonized by a potential causative agent of skeletal eroding band disease.

The first report of a successful coral disease treatment was the removal of disease-afflicted tissue from *Oscillatoria membrancea* in the Florida Keys by suctioning and applying modeling clay to cover the affected area, which was 70% effective in controlling black band disease (Hudson, 2000). A similar study performed on black band disease-afflicted *Montipora capitata* in Kauaʻi, Hawaiʻi, applied a double band of marine epoxy mixed with chlorine powder to the disease front, which significantly reduced colony mortality by 30% when compared to untreated colonies (Aeby *et al.*, 2015). Another coral disease study demonstrated that the removal of the disease front from a tissue loss affecting *Turbinaria* coral colonies in Australia halted disease progression in 80% of the treated colonies (Dalton *et al.*, 2010). Moreover, removal of growth anomalies on branching

acroporids in the Northern Line Islands displayed a 90% success rate (Williams, 2013). These methods of reducing coral morbidity and mortality, which is caused by coral disease, are a short-term solution that may allow the coral disease research community to determine the factors promoting these diseases and causative agents. The coral disease treatments published to date describe potential measures that can limit and contain the spread of newly emerging coral diseases in reef communities, but prevent from imminent disease outbreaks only in the short term. Thus, the need to eliminate the anthropogenic stressors that have direct impact on the corals is crucial.

The coral immune system

The two known types of immunological responses in vertebrate hosts are innate and adaptive. The innate immune system initially recognizes the invading microorganisms through pattern-recognition receptors (PRRs) (Janeway, 1989), which directly alert and activate immune cells, resulting in an inflammatory immune response (Akira *et al.*, 2006). Adaptive immunity features four characteristics: antigenic specificity, genetic diversity, self- and non-self-recognition, and immunologic memory. Corals are believed to have a simple and rather primitive immune system (Alker *et al.*, 2004; Pollock *et al.*, 2011; Toledo-Hernández *et al.*, 2013; Toledo-Hernández and Ruiz-Diaz, 2014). Studies using predicted protein sequences and structures in corals have identified potential pattern recognition receptors that bind to conserved microbe-associated molecular patterns (MAMPs). These potential pattern recognition receptors in corals have been speculated to activate an innate immune response upon recognition of MAMPs (Kvennefors *et al.*, 2008, 2010; Schwarz *et al.*, 2008; Hamada *et al.*, 2013). In addition, studies uncovered responses in corals that

resemble a humoral immune response, leading to the upregulation of the complement molecule C3, antibacterial peptides, and phenoloxidase activity along with melanization (Mydlarz and Palmer, 2011; Palmer *et al.*, 2011; Vidal-Dupiol *et al.*, 2011; Brown *et al.*, 2013), and Quistad *et al.* (2014) described a homolog of TNFα, found in the coral *Acropora digitifera*, which is a cytokine involved in triggering apoptosis.

Overall, corals possess an immune system that integrates an assortment of recognition receptors, genes that mediate the synthesis of a myriad of cytotoxic compounds, and cell responses that protect the coral from threats. Environmental stressors, such as elevated seawater temperatures due to global climate change, may affect the corals' capacity to recognize threats, express immune response-related genes, and outline the microbial community associated, ultimately shaping the fate of corals.

The coral probiotic hypothesis

With the development of PCR in 1983, an unexpected number and diversity of microbes were being discovered through the amplification of 16S ribosomal RNA genes. Many researchers were confused and referred to microbial contributions to mammalian or plant DNA samples as contamination. Soon the term hologenome was introduced to argue that these were likely not contamination, but rather essential members of the organism being studied. The inference was then that the organism's performance and abilities originate in the hologenome. Observations of the coral bleaching model system involving the bacterium *Vibrio shiloi* and the coral *Oculina patagonica* have shown that, despite the lack of an adaptive immune system in corals, *V. shiloi* can no longer infect *O. patagonica* (Ben-Haim *et al.*, 1999, 2004). This change from a formerly infectious pathogen no longer

being capable of inducing infection in the same host was attributed to the microbes living in symbiosis with the coral animal, which prompted proposal of the coral probiotic hypothesis (Reshef *et al.*, 2006). This hypothesis states that the coral animal shares symbiotic relationships with numerous metabolically active microorganisms. These relationships and the diversity and abundance of the population of microbial species can respond to changing environmental conditions, which allows the coral holobiont to adapt to the new condition. Koren and Rosenberg (Koren and Rosenberg, 2006) showed that the dominant bacterial species on *O. patagonica* were different in the summer and winter seasons, likely due to the temperature change of approximately 10˚C. Corals are continuously discharging mucus, which has been shown to contain large numbers of bacterial cells (Ritchie, 2006). To keep the bacterial cell abundance at a constant number, the replication rate of bacterial cells needs to remain roughly equal to the rate of loss (e.g. mucus secretion). Changes in these rates, due to environmental factors such as predators (Sussman *et al.*, 2003; Nugues *et al.*, 2004) or bacteriophage infections (Wilhelm and Suttle, 1999), can lead to an imbalance of this microbial equilibrium, and ultimately, to alterations of the bacterial communities present. These processes permit the coral holobiont to adapt within days or weeks to selective conditions, such as environmental stress. These rapid modifications of the bacterial composition may allow the coral host to use nutrients more efficiently or prevent colonization by opportunistic pathogens, maintaining resiliency to abrupt changes in the ecosystem.

The coral microbiota and pathobiome

Microbial communities have been shown to vary between the different

microhabitats within a colony (Bourne *et al.*, 2007; Rosenberg *et al.*, 2007; Ainsworth *et al.*, 2010; Sweet *et al.*, 2011; Bourne and Webster, 2013; Williams *et al.*, 2015; Hernandez-Agreda *et al.*, 2016), and the overall microbial community of the coral meta-organism or holobiont is thought to be species-specific within similar environments (Rohwer *et al.*, 2002; Sunagawa *et al.*, 2010). It has been proposed that the microbial diversity on coral exhibits differences between large scale geographical regions, on a smaller scale between reef locations, and between different depths on the same reef location (Bourne and Webster, 2013).

Studies have shown that the complex relationships shared among the constituents of the coral holobiont are dynamic and the health of the coral host depends on homeostasis of the holobiont (Rohwer *et al.*, 2002). Many biotic and abiotic factors have been shown to impact the composition of the microbiome. Biological events, coral life history, and changes in environmental parameters can cause the microbial community, richness, or abundance to shift, leading them susceptible to opportunistic infection (Harvell *et al.*, 1999, 2001; Bally and Garrabou, 2007; Lesser *et al.*, 2007; Mouchka *et al.*, 2010). Studies investigating differences in the microbial communities of healthy and diseased corals have shown that there are intra- and inter-colony variation in the community structure of corals (Pantos *et al.*, 2003; Barneah *et al.*, 2007; Shore-Maggio *et al.*, 2015) and these structures may experience dramatic changes before the development of disease lesions on the coral host (Pantos *et al.*, 2003; Wilson *et al.*, 2012).

Profiling of the host microbiota is a necessity and early work utilized culture-based methods and clone libraries (Cooney *et al.*, 2002; Rohwer *et al.*, 2002; Pantos *et al.*, 2003). However, these methods yielded only a few hundred sequences that only allowed for a

limited diversity resolution. With the advent of newer technologies, such as the PhyloChip 16S rRNA gene microarray and high-throughput sequencing technologies, a more comprehensive view of the coral-associated bacterial community has become available (Kellogg *et al.*, 2013; Ainsworth *et al.*, 2015; Apprill *et al.*, 2016). Hernandez-Agreda *et al.* compared the core microbiomes from 32 different high-throughput sequencing studies in corals that are available (Hernandez-Agreda *et al.*, 2017). Six of the 32 high-throughput sequencing projects investigated differences in the bacterial communities of healthy and diseased corals and employed Roche's 454 pyrosequencing or Illumina platforms (Cárdenas *et al.*, 2012; Apprill *et al.*, 2013; Sato *et al.*, 2013; Lesser and Jarett, 2014; Ng *et al.*, 2015; Meyer *et al.*, 2016). The sequencing output from these studies permitted the description of upwards of 100,000 different phylotypes for each of the analyzed coral species.

The importance of microbes in host health is documented in numerous organisms originating from both marine and terrestrial ecosystems. Several etiological agents of coral diseases have been identified to date (Ben-Haim and Rosenberg, 2002; Patterson *et al.*, 2002; Denner *et al.*, 2003; Sussman *et al.*, 2008; Ushijima *et al.*, 2012, 2014, 2016), and research efforts are continuing to identify more of the microbial agents responsible for the decline of global of coral reefs. Nonetheless, the rapid development and improvement of next generation sequencing technologies facilitate the identification of the microbial communities of healthy and diseased organisms. The recent findings of infectious agent studies have demonstrated that Koch's postulates have their limits (Vayssier-Taussat *et al.*, 2014). It is proposed that the pathogens live and interact with the local microbiota and are essential in the participation and generation of complex interactions, allowing for the

development of a disease process (Chow *et al.*, 2011; Vayssier-Taussat *et al.*, 2014). Therefore, the research focus should lie in the description of the "pathobiome" rather than focusing on a single bacterium, because the pathogenicity of a causative agent often relies on the strong, and quite possibly, highly specific interactions within its biotic environment (Sweet and Bulling, 2017).

Disease has emerged as a global threat to marine ecosystems and is projected to intensify in the coming decades (Altizer *et al.*, 2013; Caldwell *et al.*, 2016). It is therefore of great importance to understand the parameters that support the health and integrity of the coral animal.

Current work

This dissertation includes research on the processes involved in *Montipora* White Syndrome (MWS), a tissue loss disease that has decimated populations of *Montipora capitata,* a major reef-building coral in Kāneʻohe Bay, Hawaiʻi. Two types of MWS infections have been documented; a progressive infection that displays diffuse tissue loss termed chronic MWS (cMWS; Fig. 1), and a comparatively faster tissue loss disease termed acute MWS (aMWS; Fig. 2). Due to the slow rate of tissue loss progression, lesions of cMWS typically have algal growth directly adjacent to the disease lesion front. Prevalence of cMWS ranges from 0.02 to 0.87 % and does not exhibit seasonality. Complete colony mortality was observed to be 28 % over a period of two years, however, one third of affected colonies displayed regrowth of tissue and disease progression stopped altogether during the study period in some cases. In contrast, aMWS-infected colonies can die within days to weeks post-infection during outbreaks. In March 2010 and January 2012, two

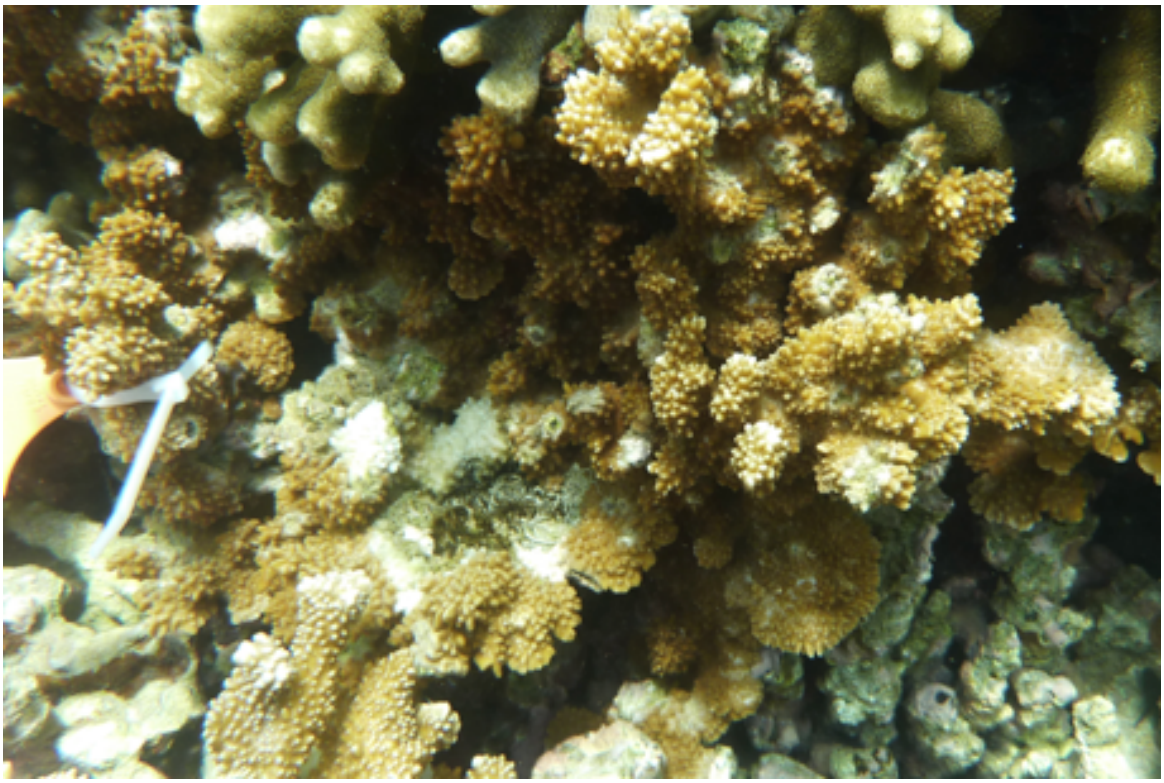

Fig. 1: *Montipora capitata* colony affected by chronic tissue loss (cMWS). Small cMWS lesions exposing the white calcium carbonate skeleton are rapidly covered by algal growth. The healthy coral tissue is orange/brown in color.

widespread and severe aMWS outbreaks were recorded on reefs throughout Kāneʻohe Bay. Surveys in 2010 and 2012 revealed 338 and 1,232 aMWS-affected *M. capitata* colonies, respectively. Disease transmission was observed between neighboring coral colonies in the field. The synchronous appearance of aMWS-afflicted colonies on multiple reefs across Kāneʻohe Bay suggested a common underlying factor in addition to the etiological agent(s).

Although very few causal relationships linking pathogens to a coral disease have been successfully demonstrated to date, two pathogenic *Vibrio* species have been shown to act as causative agents of cMWS and aMWS in *M. capitata*, *Vibrio owensii* strain OCN002 and *Vibrio coralliilyticus* strain OCN008, respectively. During controlled laboratory

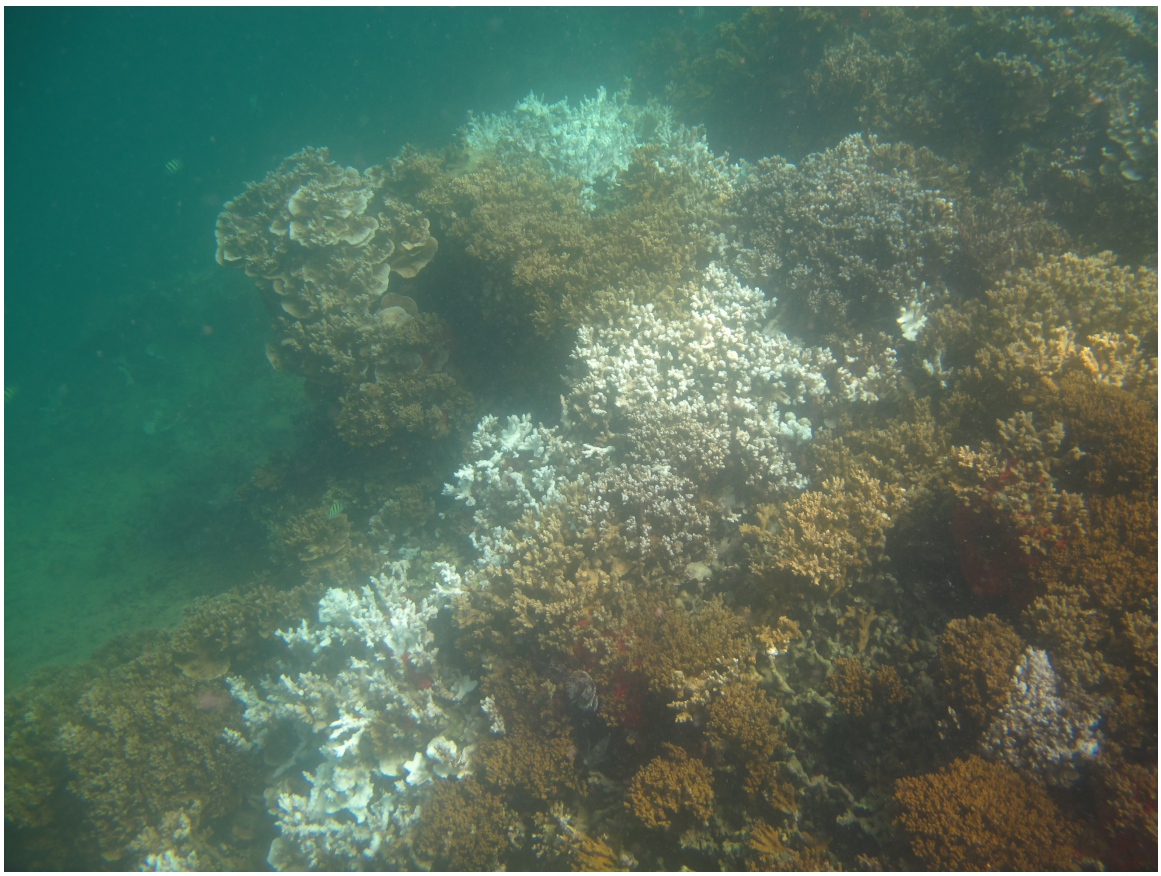

Fig. 2: Reef in Kāneʻohe Bay with multiple *Montipora capitata* colonies affected by acute tissue loss (aMWS). Coral colonies with aMWS appear bright white due to the exposed calcium carbonate skeleton. Orange/brown colonies represent healthy *M. capitata* colonies without acute tissue loss.

infections of *M. capitata*, OCN002 caused cMWS lesions in 53% of inoculated coral fragments in an average of 28 days post-inoculation, while OCN008 infected 80 - 100% of fragments with aMWS within four days post-inoculation. During infection trials and in the field, colonies exhibiting cMWS were observed to spontaneously switch to aMWS. The destructive nature of aMWS and the ability of cMWS lesions to switch to aMWS present a serious threat to the survival of *M. capitata* in Kāneʻohe Bay. The identification of novel

aMWS-causing agents and studying their mechanisms of infection need to be undertaken so effective management methods can be developed.

This dissertation investigates acute *Montipora* White Syndrome utilizing a multi-step approach. First, the bacterial contributions to aMWS susceptibility, community shifts following infection, and the bacterial contributions to aMWS during and after an aMWS outbreak were assessed. Secondly, the removal of disease lesions was assessed as a potential method of disease treatment to reduce morbidity and mortality from tissue loss associated with cMWS. And thirdly, a bacterial species, *Pseudoalteromonas piratica*, with the ability to induce tissue loss in *M. capitata* was described. Lastly, the genetic, phenotypic, phylogenetic, and biochemical characteristics of *P. piratica* strain OCN003 were described and proposed as a novel species of the genus *Pseudoalteromonas*.

CHAPTER 2: BACTERIAL COMMUNITY DYNAMICS OF HEALTHY AND DISEASED *MONTIPORA CAPITATA* COLONIES DURING AND AFTER AN ACUTE *MONTIPORA* WHITE SYNDROME OUTBREAK

INTRODUCTION

Reef-building corals are a multi-organismal association, referred to as the coral holobiont, which are comprised of the heterotrophic coral animal, endosymbiotic photosynthetic algae (*Symbiodinium* spp.), and a suite of microorganisms that include bacteria (Rohwer *et al.*, 2001, 2002; Bourne and Munn, 2005), archaea (Kellogg, 2004; Wegley *et al.*, 2004; Siboni *et al.*, 2008), viruses (Patten *et al.*, 2008), fungi (Oppen *et al.*, 2009), and protozoa (Aladro-Lubel and Martínez-Murillo, 1999; Rohwer and Kelley, 2004). These constituents form complex and diverse relationships that influence nutrition, protection, growth and fecundity (Hoegh-Guldberg, 1999). The microbiota within the holobiont have been shown to differ at nearly all scales of comparison, from different geographic regions to various locations across a single reef and different depths on the same reef location (Bourne and Webster, 2013). On an even finer scale, coral-associated microbial communities differ between the microhabitats within a colony, including the surface mucus layer, the tissues, and the skeleton (Bourne *et al.*, 2007; Rosenberg *et al.*, 2007; Ainsworth *et al.*, 2010; Sweet *et al.*, 2011; Bourne and Webster, 2013; Williams *et al.*, 2015; Hernandez-Agreda *et al.*, 2016). Despite the described spatial differences, bacterial communities from the same coral species are thought to be similar on a temporal and geographical scale (Rohwer *et al.*, 2002; Sunagawa *et al.*, 2010). These microbial

interactions suggest that the coral holobiont is not simply a set group of microorganisms, but a dynamic relationship that may change in response to the needs of the holobiont, environmental pressures, or the onset of disease.

Coral disease is a growing threat to the survival of reefs, and incidences of coral disease outbreaks are increasing worldwide (Goldberg and Wilkinson, 2004; Bourne, 2005; Aeby *et al.*, 2016). Environmental stressors, such as elevated seawater temperatures, nutrient input due to runoff, and sedimentation, have increased mortality and loss of coral cover by intensifying the effects of coral disease (Harvell *et al.*, 2007; Dalton, 2010; Haapkylä *et al.*, 2011). While research efforts are focused on identifying the microbial agents responsible for the global decline in coral reefs, only a few etiological agents of coral disease have been described to date (Ben-Haim and Rosenberg, 2002; Patterson *et al.*, 2002; Denner *et al.*, 2003; Sussman *et al.*, 2008; Ushijima *et al.*, 2012, 2014, 2016). Moreover, it is hypothesized that coral diseases arise from environmental stressors that promote proliferation of certain members of the bacterial community (i.e. opportunistic pathogens) rather than a single primary pathogen (Lesser *et al.*, 2007; Muller and van Woesik, 2012). As a result, research studies have begun to identify the constituents of the bacterial communities present in the coral holobiont and determine whether there is a change in the abundance of individual community members in healthy and diseased coral colonies.

Profiling of the host microbiota has historically utilized culture-based methods and clone libraries to identify constituents (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Pantos *et al.*, 2003). However, these methods usually only yielded between a few hundred to a thousand sequences, which merely presented a selection of the actual bacterial diversity

present. Newer technologies, such as PhyloChip 16S rRNA gene microarray and high-throughput sequencing technologies (Cárdenas *et al.*, 2012; Kellogg *et al.*, 2013; Meyer *et al.*, 2014), allow thousands to millions of individual sequences to be read from a single sample, which has made a more comprehensive view of the coral-associated bacterial community possible. In the recent review by Hernandez-Agreda *et al.* (2017), the core microbiomes from 32 different high-throughput sequencing studies in corals were compared. Six of the 32 high-throughput sequencing projects investigated differences in the bacterial communities of healthy and diseased corals (Cárdenas *et al.*, 2012; Apprill *et al.*, 2013; Sato *et al.*, 2013; Lesser and Jarett, 2014; Ng *et al.*, 2015; Meyer *et al.*, 2016). Cárdenas *et al.* (2012) compared healthy and white plague disease (WPD) affected tissue in *Siderastrea siderea* and *Diploria strigosa*. While healthy *S. siderea* exhibited a higher bacterial diversity compared to WPD-affected *S. siderea*, the opposite was observed in *D. strigosa*. Proteobacteria was the dominant phylum in all samples from both coral species, with differences in relative abundance between healthy and WPD-affected corals (Cárdenas *et al.*, 2012). The bacterial community structure of healthy *Herpolitha limax* and *Ctenactis crassa* colonies, and those displaying Yellow band disease lesions were highly variable. Gammaproteobacteria was the dominating class in *C. crassa,* whereas both Gammaproteobacteria and Alphaproteobacteria were abundant on *H. limax*. The bacterial community structure was not different between health states, but varied between reef locations (Apprill *et al.*, 2013). Another study sequenced bacterial communities present on healthy and White pox infected *Acropora palmata* colonies and found that their samples were dominated by Gammaproteobacteria. The bacterial community structure in all samples was highly variable but not significantly different between health states (Lesser

and Jarett, 2014). Meyer *et al.* (2016) evaluated whether Dark spot syndrome (DSS) is a precursor of Black band disease (BBD) in *Orbicella annularis* and *O. faveolata*. The bacterial communities of BBD-afflicted fragments were different compared to healthy and DSS-exhibiting fragments. Moreover, the relative abundance did not show differences between healthy and DSS lesion samples. Altogether, these study results demonstrate that members of the phylum Proteobacteria are the major constituents of the bacterial communities in corals. Moreover, onset of disease is usually reflected by perturbations in the bacterial community composition, possibly exacerbating disease progression.

The sequencing output from these studies resulted in millions of sequencing reads, which permitted the description of upwards of 100,000 different phylotypes for each of the coral species analyzed. Nonetheless, these methods are limited to sequencing only partial 16S rRNA gene libraries (~150-300 bp). Longer sequences (~1400-1500 bp) are needed to confidently identify abundant sequences to the species level, especially for genera that share high 16S rRNA gene sequence similarity (e.g. *Vibrio* (Haley *et al.*, 2010)). The Pacific Biosciences platform, PacBio RSII, facilitates sequencing of the full-length 16S rRNA gene, which was used to assess the bacterial communities of healthy and diseased coral in this study.

Montipora white syndrome (MWS) is a tissue loss disease that has affected populations of *Montipora capitata* in Kāneʻohe Bay, Hawaiʻi. Two types of MWS have been documented; a chronic progressive tissue loss disease termed chronic MWS (cMWS), and a comparatively faster infection termed acute MWS (aMWS), which has decimated *M. capitata* populations in Kāneʻohe Bay (Ushijima *et al.*, 2012; Aeby *et al.*, 2016). Two outbreaks of aMWS have been reported in 2010 and 2012 have led to significant coral

mortality (Aeby *et al.*, 2016). Prior surveys found few colonies afflicted by aMWS, thus, the high number of infected colonies found during these outbreaks supported its classification as an emerging disease. Two bacterial isolates, *Vibrio coralliilyticus* strain OCN008 and *Pseudoalteromonas piratica* strain OCN003 (Ushijima *et al.*, 2014, Beurmann *et al., in press*), induce acute tissue loss in *M. capitata* in laboratory infection experiments. To assess the bacterial community in *M. capitata* colonies during and after aMWS infection, DNA was extracted from samples of healthy and diseased *M. capitata* during and after an outbreak event. Full-length 16S rRNA genes were amplified, sequenced, and analyzed using the PacBio RSII high-throughput sequencing platform. The sequencing data revealed significant differences in the bacterial communities across two comparisons: between healthy and diseased *M. capitata* colonies, and *M. capitata* colonies from an outbreak and non-outbreak period. Furthermore, sequences identical to those of the two etiological agents of *M. capitata* were found in increased abundances in diseased coral samples.

RESULTS

Non-outbreak samples had more bacterial phyla in comparison to the outbreak samples.

To compare the bacterial communities in healthy and aMWS-afflicted *M. capitata* colonies, healthy fragments from apparently healthy *M. capitata* colonies (no visible tissue loss was observed on the entire colony, designated HH); healthy fragments from aMWS-afflicted *M. capitata* colonies (no tissue loss was present on collected fragments, designated HD); and fragments with acute tissue loss from aMWS-afflicted *M. capitata* colonies

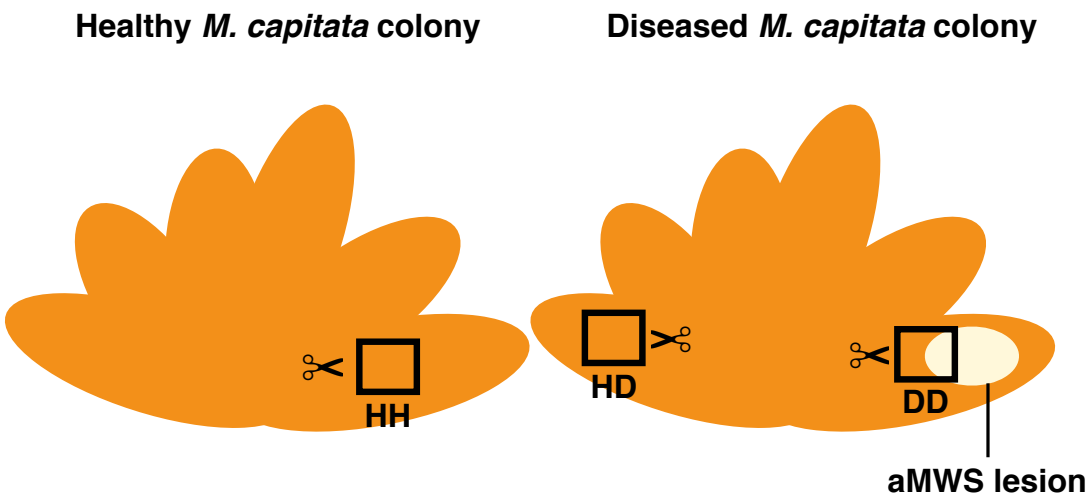

Fig. 3: Three different health states of *M. capitata* were sampled for this study. Healthy fragments of apparently healthy (= no visible tissue loss on entire colony) *M. capitata* colonies (n = 8), designated HH; healthy fragments of aMWS-afflicted *M. capitata* colonies (= no tissue loss on collected fragments; n = 8), designated HD; and fragments with acute tissue loss from aMWS-afflicted *M. capitata* colonies (= collected fragments display a section of an aMWS disease front; n = 8), designated DD.

(collected fragments displayed a section of an aMWS disease front, designated DD) were collected (Fig. 3). HH, HD, and DD samples were collected from the same sites during the 2012 aMWS outbreak (designated "outbreak") and during the 2016 non-outbreak period (designated "non-outbreak"). A total of 21 16S rRNA gene sequence amplicon libraries were created from tissue samples from healthy colonies (outbreak HH, n = 7), healthy tissue from diseased colonies (outbreak HD, n = 7), and the tissue adjacent to a disease lesion (outbreak DD, n = 7) for the 2012 aMWS outbreak sample collection. For the 2016 non-outbreak sample collection, 24 16S rRNA gene sequence amplicon libraries were generated from samples from healthy colonies (non-outbreak HH, n = 8), healthy tissue from diseased colonies (non-outbreak HD, n = 8), and the tissue adjacent to a disease lesion

(non-outbreak DD, *n* = 8). The resulting sequences were grouped based on the coral samples from which they were derived. The barcodes and primer sequences were trimmed, followed by the removal of low-quality and chimeric reads. In total, 139,484 high-quality full-length 16S rRNA gene sequences were obtained from all 45 coral samples with an average length of 1452 bp. For the 2012 and 2016 samples, 95,614 and 43,870 sequences were analyzed, respectively. The total pool of sequences was clustered into 2,850 operational taxonomic units (OTUs) at the level of 99 % identity. Outbreak samples had 2,245 OTUs present, and non-outbreak samples had 1,534 OTUs present. Overall, there was a 37.3 % (838 OTUs) overlap between the HH, HD, and DD outbreak communities, while the HH, HD, and DD non-outbreak communities had a 11.1 % (170 OTUs) overlap. A pairwise comparison between outbreak and non-outbreak periods of the communities from each of the three health states (HH, HD, and DD) showed that HH communities were made up of 2,116 OTUs with 20.2 % (428 OTUs) overlap, HD communities of 1,994 OTUs with 20.6 % (411 OTUs) overlap, and DD communities of 2,050 OTUs with 9.7 % (199 OTUs) overlap. Overall, the bacterial communities from all samples collected during both periods represented 37 phyla, 89 classes, 174 orders, 317 families, and 635 genera. Twenty-two phyla had no representation in the outbreak communities, whereas all 37 phyla were represented in the non-outbreak communities. This analysis suggests that community diversity in non-outbreak samples is generally higher than communities derived from outbreak samples.

Members of the phylum Proteobacteria dominate all samples.

The bacterial communities associated with HH, HD, and DD samples from outbreak

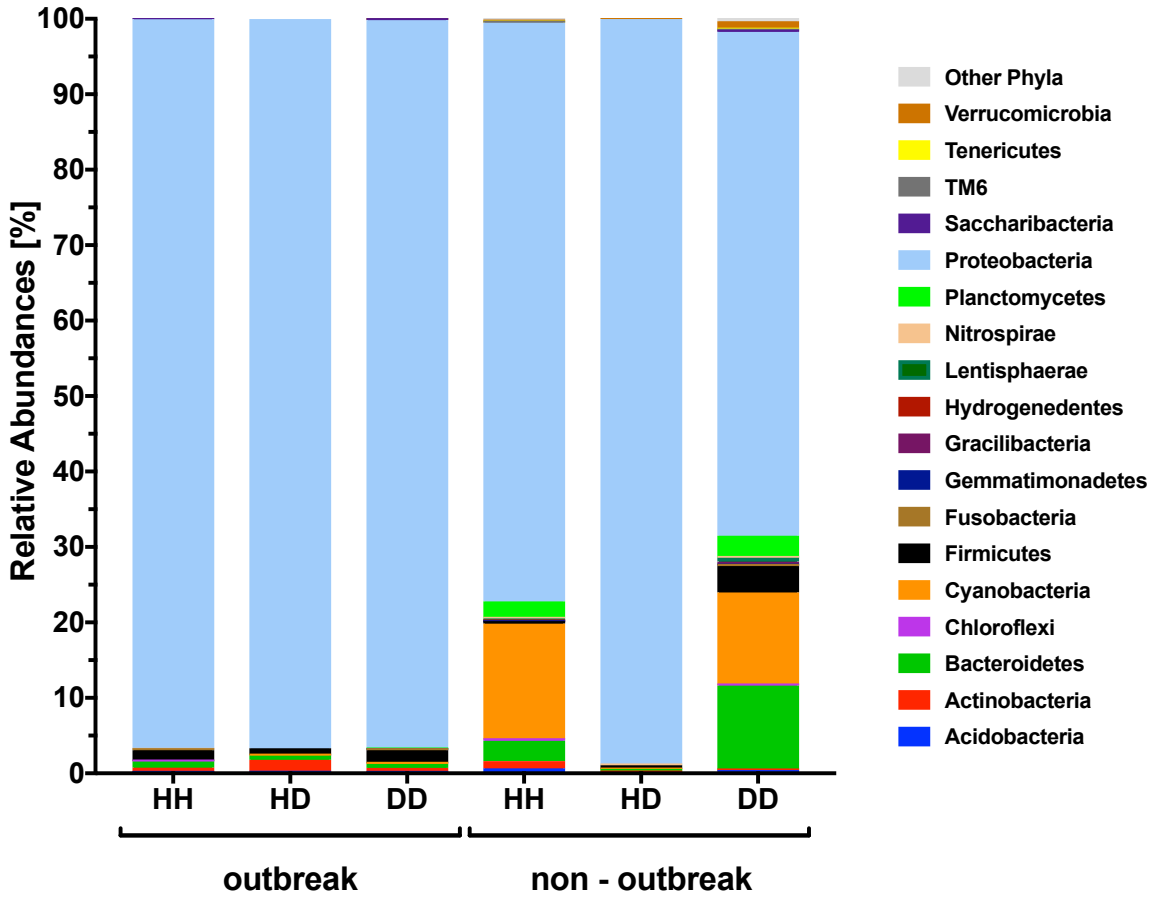

Fig. 4: Relative diversity within bacterial phyla associated with HH, HD, and DD *M. capitata* colonies from an outbreak and non-outbreak period. The 18 phyla shown were those representing greater than 0.04 % of at least one sample. The remaining phyla are collectively represented by the "Other Phyla" category. The size of each color block (assigned to phyla in the legend on the right) represents the number of sequences detected in the phyla relative to the total number of sequences detected in that sample.

and non-outbreak periods contained members of common marine and coral-associated bacterial phyla including the Proteobacteria, Cyanobacteria, Bacteroidetes, and Firmicutes (Wilson *et al.*, 2012) (Fig. 4). Proteobacteria dominated all libraries from the outbreak samples evenly (96.6 - 96.8 %). Libraries from the HH and DD non-outbreak samples

displayed a lower abundance of Proteobacteria sequences (66.8 % and 76.7 %, respectively), while this phylum was highly represented in the HD samples (98.9 %). The proportion of Proteobacteria in the outbreak samples is significantly lower than in the non-outbreak samples (Mann-Whitney, $U = 142$, $n_1 = 21$, $n_2 = 24$, $p = 0.012$), which suggests that different bacterial community structures are associated with coral colonies during outbreak and non-outbreak periods.

Abundance of Enterobacteriales significantly decreased in diseased samples from the non-outbreak samples.

More bacterial orders within the Proteobacteria phylum are represented in the non-outbreak samples than the outbreak samples (Fig. 5). Sequences from the order Enterobacteriales dominated all HH and HD outbreak and non-outbreak samples (ranging from 80 to 96.8 %). The DD non-outbreak samples showed a significantly lower number of Enterobacteriales than did DD samples from the outbreak (16.1 % and 80 %, respectively; Mann-Whitney, $U = 4$, $n_1 = 7$, $n_2 = 8$, $p = 0.004$). Additionally, all DD samples from outbreak and non-outbreak periods had a high abundance of Rhodobacterales (14.5 % and 33.8 %, respectively). Oceanospirillales, Cellbirionales, Vibrionales, and Rickettsiales display significantly high abundances in the DD samples collected during the non-outbreak period (11 %, 7.4 %, 3.7 %, and 1.8 %; respectively). There were significantly more representatives from the order Alteromonadales in the non-outbreak samples (2.9 % to 5.3 %) than the outbreak samples (<0.1 %; Mann-Whitney, $U = 51.5$, $n_1 = 24$, $n_2 = 21$, $p < 0.001$), while sequences from the Burkholderiales were in significantly higher proportions in the outbreak samples (0.6 % to 1.2 %) compared to non-outbreak samples (< 0.03 %; Mann-

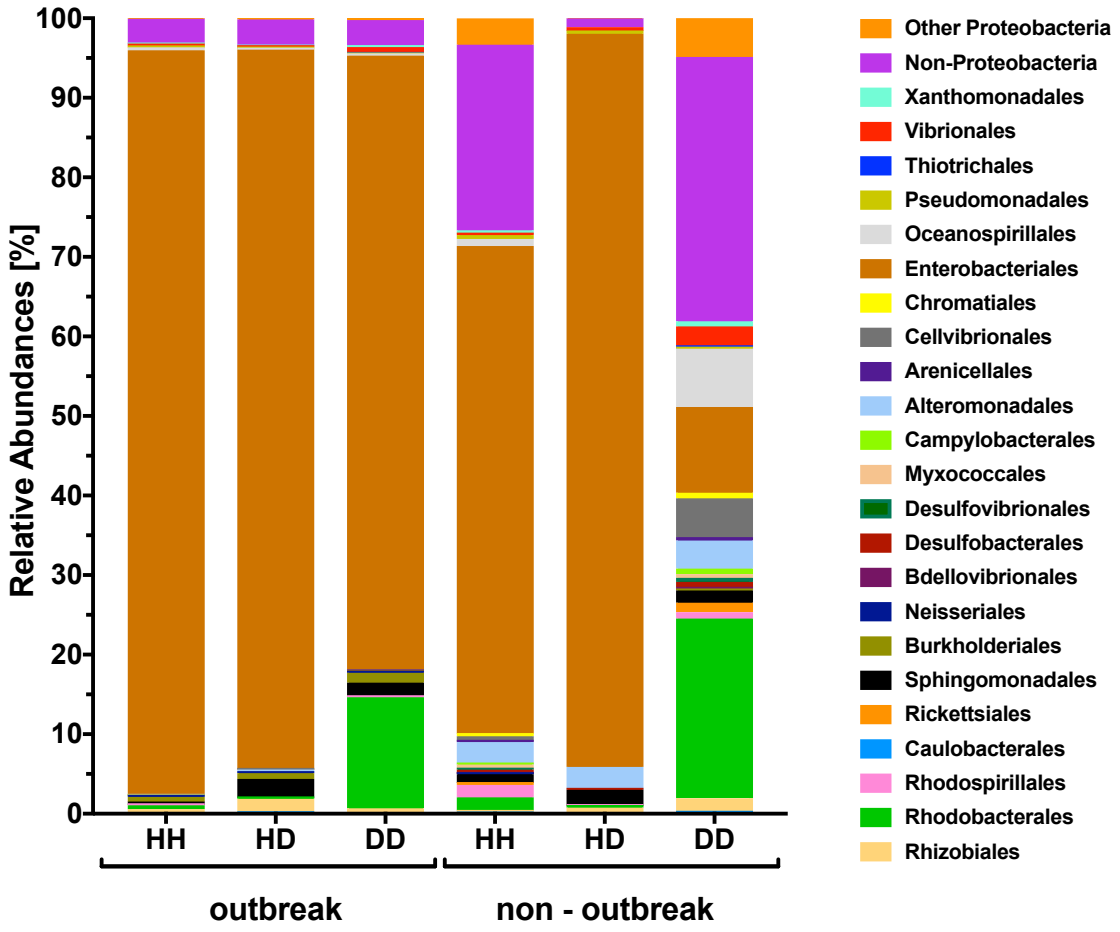

Fig. 5: Relative diversity within bacterial orders within the phylum Proteobacteria associated with HH, HD, and DD *M. capitata* colonies from an outbreak and non-outbreak period. The 23 orders shown were those representing greater than 0.03 % of at least one sample. The remaining orders within the Proteobacteria are collectively represented by the "Other Proteobacteria" category, and the orders that were from other phyla are represented within the "Non-Proteobacteria" cateogry. The size of each color block (assigned to orders in the legend on the right) represents the number of sequences detected in the order relative to the total number of sequences detected in that sample.

Whitney, $U = 15$, $n_1 = 24$, $n_2 = 21$, $p < 0.001$). A significant increase in the proportion of Cyanobacteria, Bacteroidetes, and Planctomycetes was observed in the non-outbreak compared to the outbreak samples (Mann-Whitney, $U = 48$, $n_1 = 21$, $n_2 = 24$, $p < 0.001$; $U =$

148.5, n_1 = 21, n_2 = 24, p = 0.018; and U = 150, n_1 = 21, n_2 = 24, p = 0.008, respectively). Firmicutes displayed a significantly increased abundance in the non-outbreak samples compared to the outbreak samples (Mann-Whitney, U = 99, n_1 = 21, n_2 = 24, p < 0.001). These data indicate that Proteobacteria generally dominated all samples regardless of outbreak status, and the Cyanobacteria, Bacteroidetes, and Planctomycetes phyla were more abundant in non-outbreak samples than outbreak samples.

Table 1: Summary statistics and Shannon–Wiener diversity indices for bacterial communities retrieved from *M. capitata* fragments. Samples were taken from colonies that showed no signs of disease (HH), coral fragments taken from an area of apparently healthy tissue on the diseased colonies (HD), and coral fragments taken from an area of diseased tissue on the diseased colonies (DD) during an outbreak of aMWS and a non-outbreak period.

Sample ID	Total number of reads	Species richness	Total reads subsampled	Species richness	Species diversity	Species evenness
HH_outbreak	30738	1622	12000	963	2.21	0.74
HD_outbreak	33440	1610	12000	949	2.22	0.75
DD_outbreak	31436	1622	12000	948	2.31	0.78
HH_non-outbreak	16133	922	12000	799	2.50	0.86
HD_non-outbreak	12323	795	12000	786	2.59	0.89
DD_non-outbreak	15414	647	12000	596	2.08	0.75

Higher bacterial diversity and evenness was observed in the outbreak samples.

The total diversity and evenness of the bacterial communities present in the samples collected during the outbreak are significantly different than those from a non-outbreak period with an overall higher diversity and evenness in non-outbreak samples (Table 1; Mann-Whitney, U = 125.5, n_1 = 21, n_2 = 24, p = 0.010; U = 109.5, n_1 = 21, n_2 = 24, p = 0.003, respectively). However, there is not a significant difference in total species richness between the samples from the outbreak and non-outbreak period (Mann-Whitney, U =

195.5, $n_1 = 21$, $n_2 = 24$, $p = 0.40$). This implies that the difference in total diversity is due to the unequal numerical representation of sequences for each species.

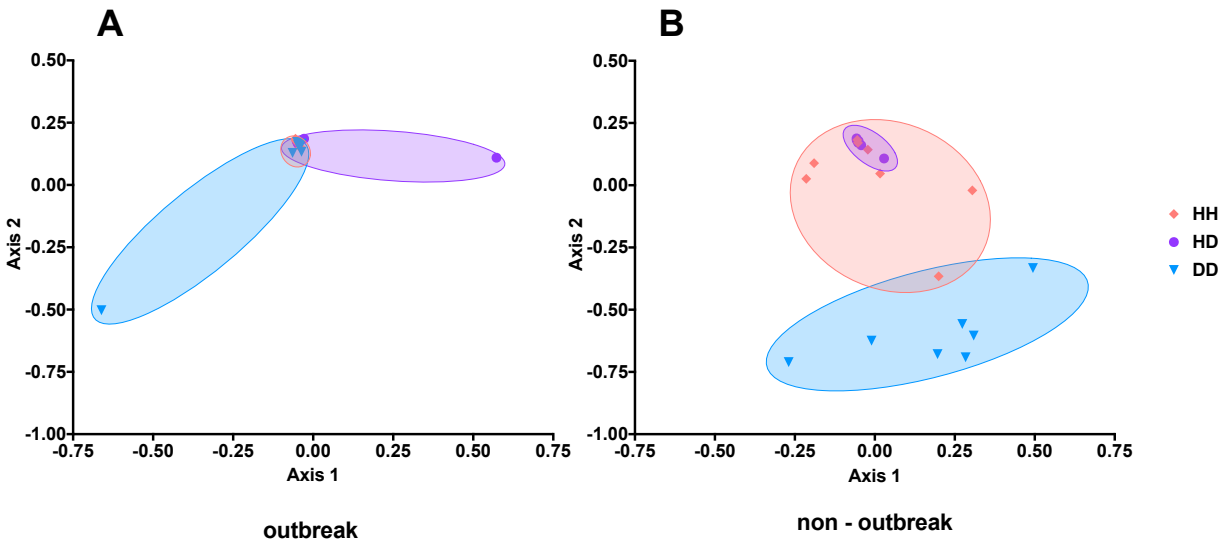

Fig. 6: Non-metric multidimensional scaling (NMDS) plot of samples, based on the majorization algorithm (Borg and Groenen, 1997). The lowest stress value was 0.09 with an R^2 value of 0.99, which reflects the high quality of the ordination. (A) Bacterial communities of HH, HD, and DD samples during the outbreak of aMWS in 2012. (B) Bacterial communities of HH, HD, and DD samples during the non-outbreak period in 2016.

The community structure of samples collected during an aMWS outbreak and a non-outbreak period are significantly different.

When comparing the individual bacterial community structures, the outbreak samples and non-outbreak samples show significant differences, as determined by ANOSIM (Global $R = 0.265$, $p < 0.001$). The non-dimensional scaling (NMDS) plot indicates that the community structure from HH, HD, and DD outbreak samples cluster tightly, except for one HD and one DD sample (Fig. 6A). ANOSIM of the outbreak communities suggests that they

share similar variances (HH-HD: $R = 0.008$, $p = 0.349$, HD-DD: $R = 0.032$, $p = 0.221$, HH-DD: $R = 0.101$, $p = 0.092$). NMDS analysis of the non-outbreak samples shows that the HH communities cluster tightly with the HD communities, but group away from the DD communities (Fig. 6B). This finding is supported by the multivariate data analysis ANOSIM, reporting statistical significance between the HD and DD, and between the HH and DD non-outbreak community variances (HD-DD: $R = 0.991$, $p < 0.001$, HH-DD: $R = 0.863$, $p < 0.001$). A weighted UniFrac analysis, which takes the relative relatedness of community members and their abundances into account, confirmed that significant differences exist between communities within the outbreak and non-outbreak samples, as well as among the same health states of outbreak and non-outbreak samples (e.g. HH samples from the outbreak and HH samples form the non-outbreak period). Altogether, bacterial community differences between health states of *M. capitata* were observed for both outbreak and non-outbreak samples. However, less variance was evident within the outbreak communities when compared to the non-outbreak communities.

Escherichia spp. contribute to the overall dissimilarity of the microbiota.

To determine which OTUs contribute most to the total dissimilarity between the communities that displayed significant ANOSIM results, a SIMPER analysis was conducted (Table 3, at the end of this chapter). The OTUs that contributed most to differences between HH versus DD and HD versus DD communities from the non-outbreak period were classified as *Escherichia* (20.16 % and 12.54 % of total dissimilarity [OTD], respectively), *Shimia* (3.02 % and 3.48 % OTD, respectively), and *Nautella* (2.75 % and 2.84 % OTD, respectively). The OTUs that contributed most to the dissimilarity in the HH

communities between the outbreak and non-outbreak samples were *Escherichia* (30.48 % OTD), *Shimia* (5.35 % OTD), and *Sphingomonas* (3.12 % OTD). In contrast, the OTUs with the highest contribution to the differences of DD communities between the outbreak and non-outbreak samples were *Escherichia* (29.24 % OTD), *Shimia* (4.97 % OTD), and *Nautella* (3.78 % OTD). These data implicate representatives of the genus *Escherichia* in contributing to the overall dissimilarity of bacterial communities between healthy and diseased coral in this study.

In silico bacterial source tracking suggests the fecal coliform to be of livestock origin.

The results of the SIMPER analysis revealed that *Escherichia* spp. contribute to the overall dissimilarity of the bacterial communities of healthy and diseased *M. capitata* colonies during and after an aMWS outbreak. Members of the genus *Escherichia* are commonly found in the gastrointestinal tract of warm-blooded animals, such as humans and livestock (Ishii and Sadowsky, 2008). To identify the origin of the fecal coliform sequences (*Escherichia* spp.) present in this analysis, bacterial source tracking was conducted with representative sequences from OTUs of the order Bacteroidales, which correlate with fecal coliforms (Okabe and Shimazu, 2007). The cow-, pig-, and human-specific *Bacteroides-Prevotella* 16S rRNA gene sequences described in Okabe and Shimazu (2007) were aligned with the Bacteriodes sequences from this study, and a maximum likelihood phylogenetic tree was constructed (Fig. 7). The tree recapitulates the clustering observed by Okabe and Shimazu (2007) with the cow-specific sequences clustering between the human- and pig-specific sequences. Interestingly, the coliform OTUs from the

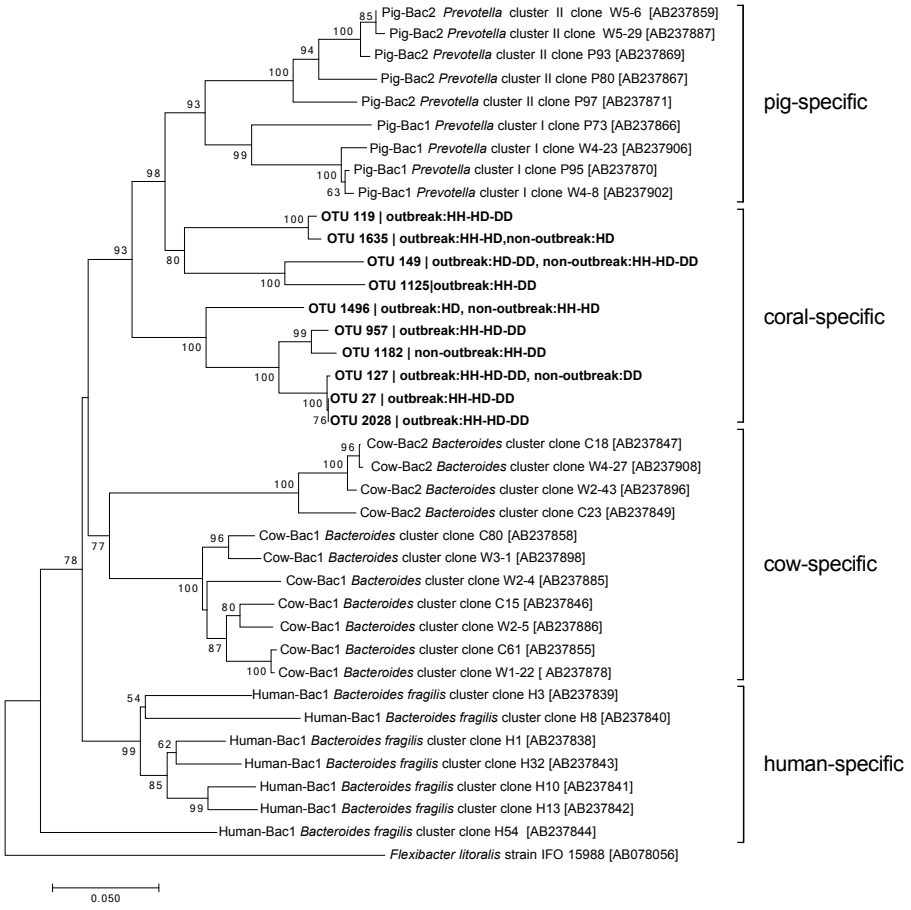

Fig. 7: Neighbor-joining phylogenetic tree derived from human-, pig-, and cow-specific *Bacteroides-Prevotella* 16S rRNA gene sequences (Okabe and Shimazu, 2007). Bacterial markers were aligned with representative sequences from OTUs of the order *Bacteroidales* from the sequencing data. *Flexibacter litoralis* strain IFO 15988 was chosen as the outgroup. The scale bar represents five nucleotide substitutions per 100 nucleotides. Bootstrap values >50% (500 replicates) are indicated at nodes. GenBank accession numbers for each reference strain are in parentheses.

M. capitata outbreak/non-outbreak samples group together to form a *M. capitata*-specific cluster. This cluster is located between the pig- and cow-specific *Bacteroides-Prevotella* clusters, suggesting closer phylogenetic relatedness to pig- and cow- specific markers than

human-specific markers. These bacterial source tracking results indicate that the origin of the *Bacteroides* sequences is likely from pigs and cows, which suggests the fecal contamination to be of animal, rather than human, origin.

A cultivable fecal coliform did not cause disease in healthy *M. capitata*.

The first example of marine "reverse zoonosis", white pox disease of *Acropora palmata* in the Florida Keys, was caused by the human pathogen *Serratia marcescens*, which originated from wastewater, and extensively reduced *A. palmata* cover in the Caribbean (Sutherland *et al.*, 2010, 2011). To investigate whether isolates of the most

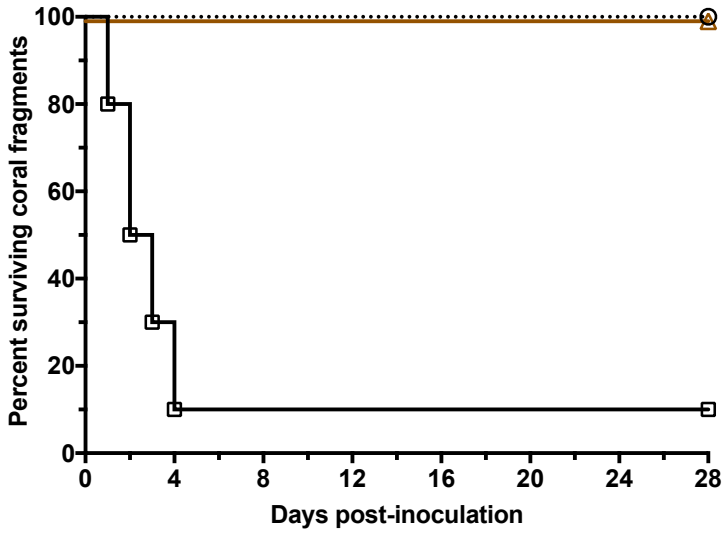

Fig. 8: Kaplan–Meier survival curve of healthy *M. capitata* fragments exposed to OCN300 (fecal coliform candidate), OCN008 (positive control; known pathogen of *M. capitata*), and OCN004 (negative control; normal microbiota of *M. capitata*), at 25˚C. The solid black line with open black squares represents fragments exposed to OCN008 (n = 8); the dashed black line with open black circles represents fragments exposed to OCN004 (n = 8); and the solid brown line with open brown triangles represents fragments exposed to OCN300 (n = 8). The concentration of bacteria was 10^8 CFU/ml of seawater.

abundant *Escherichia* OTU elicited signs of disease in healthy *M. capitata,* a total of ten lactose-fermenting bacterial isolates were cultured on enteric-selective MacConkey agar medium (one isolate from each of three different aMWS-afflicted (DD) fragments, two isolates from each of two apparently healthy (HH) fragments, and three isolates from one healthy-diseased (HD) coral fragment). One isolate from each health state was selected and identified by sequencing the full 16S rRNA gene. Comparison with sequences from the BLAST database revealed that the three isolates shared 100% sequence identity with the fecal coliform *Escherichia fergusonii.* Based on the similarity of these isolates, the remainder of the study utilized a single isolate, *E. fergusonii* strain OCN300. The 16S rRNA gene sequence of OCN300 [KY421548] was 100% identical to the most abundant OTU that made up 55.89 % of the total sequences. To test OCN300's ability to induce disease signs in healthy *M. capitata*, experimental infection trials were conducted. When utilized in infection trials (Fig. 8), OCN300 failed to induce signs of disease in any of the healthy *M. capitata* fragments, whereas the positive control, the coral pathogen *Vibrio coralliilyticus* strain OCN008 (Ushijima *et al.*, 2014), caused acute tissue loss in 87.5 % of the healthy fragments (McNemar's test: $n = 8$, $p = 0.02$). Fragments inoculated with the negative control bacterium, *Alteromonas* sp. strain OCN004, remained healthy for the duration of the experiments (28 days). To test whether the high abundance of fecal coliform influenced infection by the opportunistic *M. capitata* pathogen, *Pseudoalteromonas piratica* strain OCN003 (Beurmann *et al., in press*), fragments were pre-treated with OCN300 for three days and then exposed to OCN003. The infection rate of OCN003 as a primary pathogen was not increased due to the pre-treatment by the fecal coliform (comparison of survival curves; Mantel-Cox test: $n = 8$, $p = 1.00$). Taken together, the results indicate that OCN300

did not act as an etiological agent of MWS in laboratory experiments. Nevertheless, the effects of fecal coliform bacteria may compound the negative influence of environmental factors (i.e. decreased salinity, increased nutrients, etc.) on the health of *M. capitata*.

Previously described *M. capitata* pathogens found among most abundant OTUs.

Laboratory infection experiments demonstrated that *Vibrio coralliilyticus* strain OCN008 (Ushijima *et al.*, 2014) and *Pseudoalteromonas piratica* strain OCN003 (Beurmann *et al.*, *in press*) have the ability to induce acute tissue loss in healthy *M. capitata*. Utilizing PacBio, which sequences bacteria's full-length 16S rRNA gene sequence, permitted the screening for these previously described aMWS-inducing *M. capitata* pathogens among the bacterial community. Comparison of the OCN008 16S rRNA sequence with the most abundant *Vibrio* OTU of this analysis indicates 100 % sequence identity between these sequences. This OTU was observed in both outbreak and non-outbreak HH, HD, and DD samples. However, the number of sequences increased by two-fold in the non-outbreak DD samples (0.98 %), when compared to HH and HD (0.45 % and 0.36 %, respectively). Additionally, the most abundant OTU within the order Alteromonadales was classified as *Pseudoalteromonas* and shared 99 % full-length 16S rRNA gene sequence identity with *P. piratica*. This OTU was found in fragments from the non-outbreak period with low abundances in the HH and DD samples (0.18 % and 0.07 %, respectively). Interestingly, the increased abundance in the non-outbreak HD samples (1.16 %) suggests a change in conditions that led to the proliferation of this species of *Pseudoalteromonas*. In conclusion, strain OCN008 is found consistently in diseased *M. capitata,* whereas strain OCN003 may

be part of the transient microbiota with the ability to proliferate in an already health-compromised host.

DISCUSSION

This is the first study to compare the bacterial community structures of healthy and diseased coral on a temporal scale using full-length 16S rRNA gene sequences analyzed with the high-throughput PacBio RSII sequencing platform. The overall community structure at the phylum level resembled communities commonly found in coral reef environments, with a high overall abundance of Proteobacteria (66.8 - 98.9 % of all sequences by sample; Fig. 4; Wilson *et al.*, 2012). An *Escherichia* species was the most dominant OTU and was the greatest contributor to the overall dissimilarity between health states and sample collections. Bacterial source tracking indicated that this OTU was likely introduced into the reef following contamination with livestock, rather than human, sewage. Interestingly, infection experiments using a cultivated isolate belonging to this coliform OTU did not elicit disease signs nor promote infection in healthy *M. capitata,* suggesting it may not be a primary factor in aMWS disease development.

Previous studies describing coral associated bacteria have used high-throughput sequencing platforms that analyze <500 bp fragments (i.e. Roche 454, HiSeq Illumina, Ion Torrent). Partial 16S rRNA gene sequencing facilitates the identification of bacterial genera and, in some cases, species. However, to discriminate between closely related species (e.g. the different species within the genera *Vibrio* and *Pseudoalteromonas* (Sawabe *et al.*, 2007, Beurmann *et al., in press*), sequencing the entire 16S rRNA gene is essential. A study done by Mosher *et al.* (2014) assessed the improved P4/C2 chemistry of the PacBio RSII

platform and compared it to the older XL/C2 chemistry. The results showed an increase in sequence yield and an overall reduction in the error rate (Mosher *et al.*, 2014). The same study demonstrated that species-level identification is not possible with partial 16S rRNA gene sequence read lengths (<500 bp), as the accuracy for identification against known sequences in the NCBI database was between 36.2-67.5%, whereas the accuracy of full-length 16S rRNA gene sequence exhibited >99% accuracy at both the genus and species level (Mosher *et al.*, 2014). The PacBio RSII platform results therefore surpassed the accuracy of Roche's 454 pyrosequencing platform, and the read lengths of >1,400 bp that are achievable with PacBio RSII facilitates the identification of bacteria to the species level in environmental samples, such as healthy and diseased coral tissue (Schloss *et al.*, 2016; Wagner *et al.*, 2016).

Obtaining full-length 16S rRNA gene sequences permitted inspection of the samples to unambiguously identify bacteria similar to the previoulsy described *M. capitata* aMWS pathogens, *Vibrio coralliilyticus* strain OCN008 and *Pseudoalteromonas piratica* strain OCN003 (Ushijima *et al.*, 2014, Beurmann *et al.*, *in press*). *Vibrio coralliilyticus*, a primary pathogen of *M. capitata,* was found in higher abundances on diseased corals in both outbreak and non-outbreak samples, whereas *Pseudoalteromonas piratica*, a secondary pathogen of *M. capitata*, was found exclusively in non-outbreak samples with higher abundance in HD corals. The abundances of these pathogens may provide insight into their interactions with coral and infection strategies. The presence of *V. coralliilyticus* on healthy and diseased coral suggests that it could be a member of the normal flora that causes diseases when its numbers increase. Because strains of both *V. coralliilyticus* and *P. piratica* have been identified as etiological agents of aMWS, a disease with one disease sign, it is

impossible to determine which pathogen is the cause of aMWS on each colony in the field. Sequencing complete the 16S from the bacterial communities has provided additional insight into the disease dynamics of the above pathogens during aMWS infection.

The bacterial communities from healthy and diseased *M. capitata* during and after the disease outbreak are statistically different. The increase of copiotrophic bacterial markers, members of the orders Alteromonadales, Bacteroides, amd Firmicutes, as well as a decrease in representatives of the order Burkholderiales during the non-outbreak period implies that the aMWS outbreak in 2012 was supported by a different bacterial community configuration, and ultimately, by different aMWS pathobiomes. A similar shift is observed in the study conducted by Closek *et al.* (2014) in which the community structure dramatically changed during the transition from a healthy to a diseased state (HD and DD samples) when comparing coral colonies affected by Yellow band disease. Moreover, bacterial families that had previously been associated with coral disease, including members of Vibrionales, were found at higher abundances in diseased coral samples. *Vibrio* spp. are known to be causative agents of, or be associated with, a number of coral diseases (Kushmaro *et al.*, 1997; Ben-Haim and Rosenberg, 2002; Cervino *et al.*, 2004; Sussman *et al.*, 2008; Ushijima *et al.*, 2012, 2014, 2016). An increase of Alteromonadales and Rhodobacterales sequences in diseased coral samples have been described in Caribbean yellow band- and white plague-diseased corals (Cervino *et al.*, 2004; Sunagawa *et al.*, 2009; Meyer *et al.*, 2014). Sunagawa *et al.* (2009) hypothesized that the increase in the abundance of Rhodobacterales in white plague-afflicted corals was due to the uncontrolled growth of opportunistic commensals as a response to disease, which was similar to the response observed in this study and could be a cause or symptom of aMWS

infections. In addition, increased abundances of members of the Firmicutes, Planctomycetes, Bacteroidetes, and Cyanobacteria have also been observed in other coral diseases, such as black band diseased corals of the Red Sea and corals with lesions caused by a temperature-related disease outbreak on the Great Barrier Reef (Jones *et al.*, 2004; Barneah *et al.*, 2007). Despite the similarities of major phyla presented here to those from previous coral disease sequencing studies, this analysis revealed an unprecedented abundance of *Escherichia* sequences during and after an aMWS outbreak.

The abundance of *Escherichia* spp. in both outbreak and non-outbreak periods suggests that it may be a consistent part of the microbiome of *M. capitata* in Kāneʻohe Bay. Analysis of the stream flow rates did not exhibit significant differences in freshwater input into the bay, and was ruled out as a source of bias in the experimental process since the DD samples of the non-outbreak samples showed an almost ten-fold reduction in *Escherichia* sequences. In addition, comparison of the Kāneʻohe Bay *M. capitata* bacterial communities at the time of collection and after four days in aquaria with filtered seawater resulted in an average 50 % reduction in the abundance of *Escherichia* sequences (Ushijima *et al.,* unpublished data), suggesting persistence of these bacteria on corals in the environment. Studies investigating the residence time of *E. coli* in marine waters have shown that they survive for long periods of time and occasionally grow in numbers, especially when sediment material is present (Gerba and McLeod, 1976; Davies *et al.*, 1995). Representatives of the genus *Escherichia* are known fecal coliforms and indicators of fecal pollution (Dufour, 1977), and fecal coliforms have been found in high abundances in the vicinity of marine sewage outfall areas (Edwards *et al.*, 1998; Wear and Thurber, 2015). Kāneʻohe Bay has had a long history of fecal contamination; large quantities of sewage

were discharged into the southern bay until the sewage outfalls were diverted out of the bay in 1978 (Tanaka *et al.*, 2013; Stimson, 2015). The many bay-adjacent homes not connected to the sewer system still lead to the seepage of fecal coliform bacteria and sediment runoff into the nearshore reef environment, an event that has been proposed to cause disease in marine organisms (Grigg, 1994; Bahr *et al.*, 2015; Aeby *et al.*, 2016).

It has been hypothesized that freshwater runoff can perturb coral and make them more susceptible to infection. The bacterial coliforms originating from livestock in the above *M. capitata* samples may have entered the reef system via runoff. The combined input of freshwater and coliforms could act as an inciting factor in disease onset, especially since both the 2010 and 2012 aMWS outbreaks occurred during the rainy winter months (Aeby *et al.*, 2016). To determine whether freshwater runoff contributed to the 2010 and 2012 aMWS outbreaks, the rate of freshwater input into Kāneʻohe Bay prior to sample collection in 2012 was analyzed. The average daily stream flow into Kāneʻohe Bay during the four weeks preceding the 2012 sample collection was 1.59 m^3/s (± SD 0.85 m^3/s). This rate was compared to the average daily stream flow during the same winter month in 2016, which displayed and average daily rate of 1.76 m^3/s (± SD 0.11 m^3/s). Comparison of the average daily stream flow rates (Paired *t* test: $t(27) = 1.06$, $p = 0.30$) during the month preceding the 2012 aMWS outbreak sample collection and the 2016 non-outbreak sample collection indicates that freshwater input into Kāneʻohe Bay via nearby streams may not to be a major inciting factor in the 2010 and 2012 aMWS outbreaks.

Sewage pollution is a great concern to our health and methods designed to determine the origin of the fecal coliform contamination are receiving increasing attention as our understanding of zoonotic disease progresses (Harwood *et al.*, 2014). *In silico*

bacterial source tracking using the sequencing data obtained in this study was performed to determine the origin of the *Escherichia* sequences derived from coral. The clustering of the *Bacteroides* markers, which have been shown to correlate with fecal bacteria, indicates that the coliforms originate from livestock rather than humans (Fig. 7). Adjacent to Kāneʻohe Bay, there is a 4,000-acre cattle ranch in Kualoa, a pig farm in the Koʻolau mountains, and an unknown number of feral pigs in the forest of the Koʻolau mountain range (estimated feral pig population in the state of Hawaiʻi: 80,000; Mayer and Brisbin, 2008). Globally, coral reefs are in decline, and land-derived pollution, including sewage, are a major contributor of that deterioration. Sewage, be it of human or livestock origin, should not be considered a single stressor, but rather a compilation of multiple stressors. Freshwater, pathogens, suspended solids, inorganic nutrients, and heavy metals are just a few of the agents that impair the integrity of the coral holobiont. The synergistic combination of sewage contaminants and other stressors, such as rising seawater temperatures, have the potential to accelerate disease in the coral reef ecosystem. To mitigate this global threat, future research needs to focus on further describing the corals' tolerance to sewage exposure, the origin and extent of the sewage, and most importantly assessing management strategies that can be employed to reduce the impacts of the sewage runoff that enters the near-shore coral reef environment.

MATERIALS AND METHODS

Sample collection, processing, and environmental data.

Three different health states of *M. capitata* were sampled from colonies growing at depths of one to three meters from the fringing reef surrounding Moku O Loʻe island in

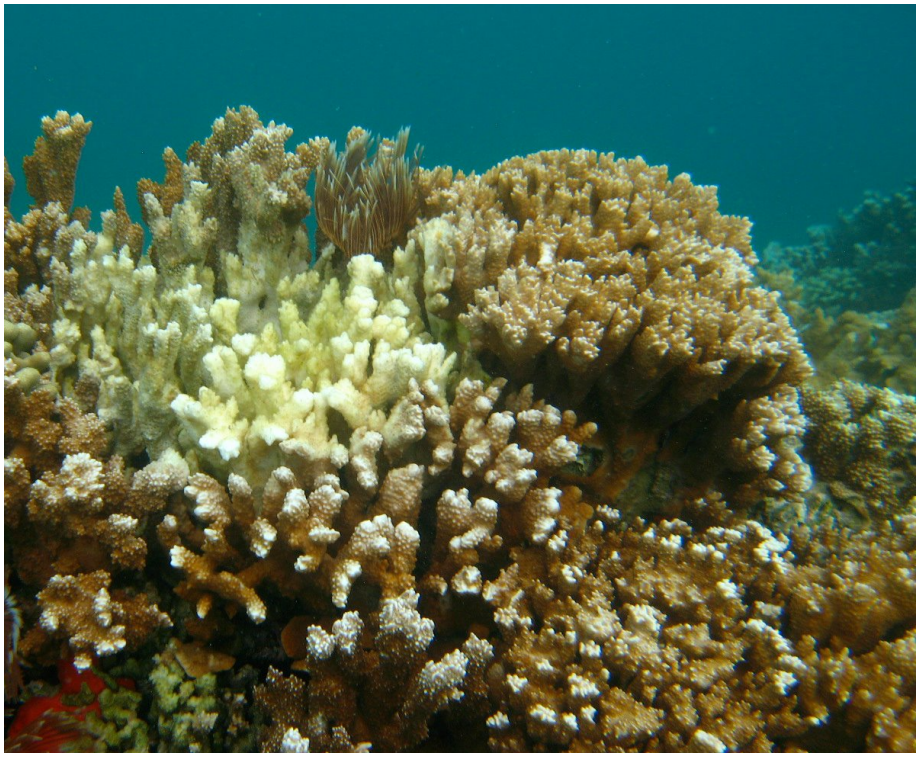

Fig. 9: *Montipora capitata* colony displaying acute *Montipora* white syndrome (aMWS; white lesion showing exposed coral skeleton next to orange, healthy tissue).

south Kāneʻohe Bay, Hawaiʻi. *M. capitata* fragments were placed into individual bags at depth and brought to the laboratory for immediate processing as previously described (Shore-Maggio *et al.*, 2015). Seven coral fragments in each of the three health states (HH, HD, and DD) were collected during the recorded aMWS outbreak in 2012 (Fig. 9; Aeby *et al.*, 2016) and eight coral fragments in each of the three health states were collected during a non-outbreak period in 2016 (n = 21 and n = 24 for 2012 and 2016, respectively). The same location on the reef was used for both sample collections to control for environmenta l conditions (Fig. 10). All coral fragments used in this study were collected under Special Activities Permits SAP#2011–68 and SAP#2015-48 granted by the State of Hawaiʻi, Department of Land and Natural Resources, Division of Aquatic Resources.

To measure and assess stream flow, data was collected from five USGS stream

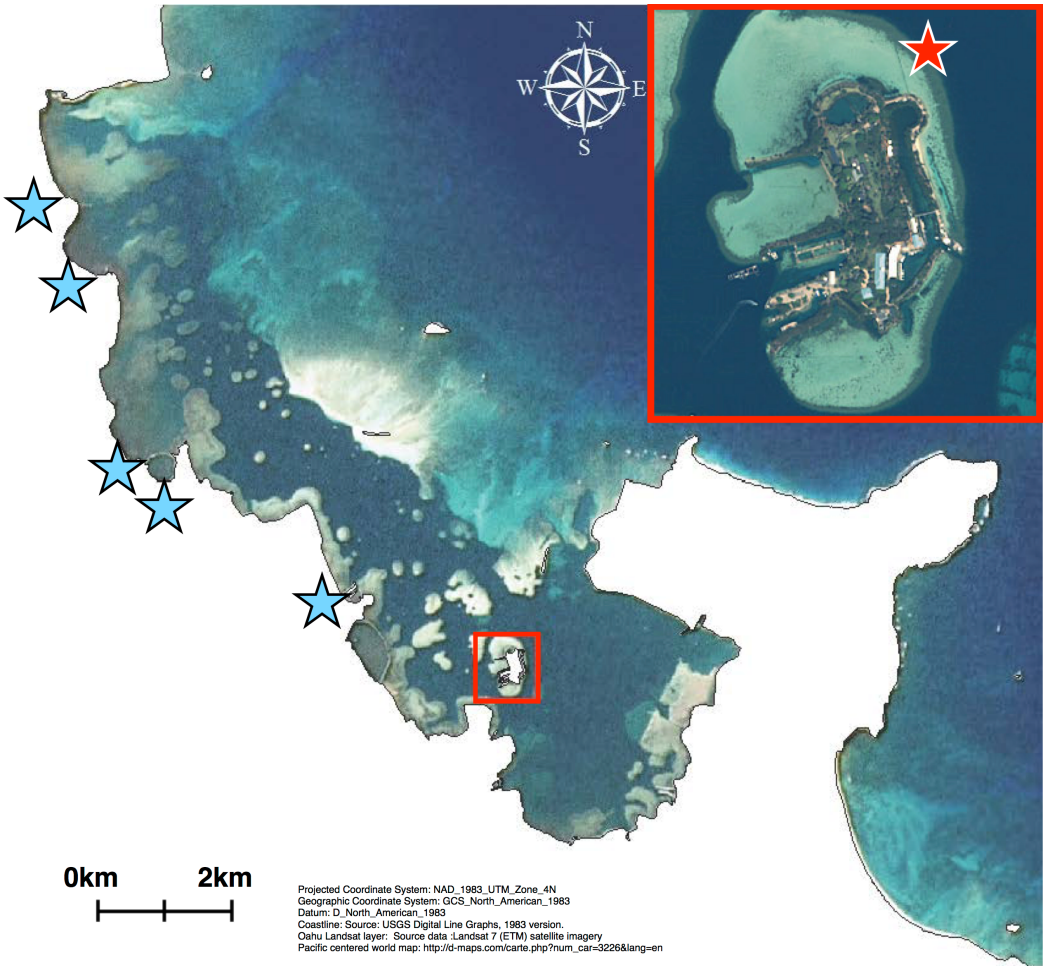

Fig. 10: Map of Kāneʻohe Bay, Oʻahu, with the location of Moku o Loʻe island (red box), enlarged as insert. The location of the sampling site (around Moku o Loʻe island) is denoted by a red asterisk. The location of the five stream gauges are denoted by blue asteriks.

gauges at Kāneʻohe Bay (Fig. 10; #16283200 Kahaluʻu Stream, #16284200 Waiheʻe Stream, #16294100 Waiāhole Stream, #16294900 Waikane Stream, #16275000 Heʻeia Stream; available at http://maps.waterdata.usgs.gov/mapper/index.html). The mean daily flow rates from the four weeks preceding coral sample collections were compared and statistically analyzed using a paired *t* test.

DNA extraction and PCR amplification.

To compare the bacterial communities of HH, HD, and DD fragments during and after an aMWS outbreak, total DNA was extracted from each isolate using the MoBio PowerSoil DNA extraction kit (MoBio Carlsbad, CA, USA) according to the manufacturer's instructions with minor modifications. Approximately 10 g of each coral fragment was crushed in 10 ml of filtered seawater (FSW). The crushate was allowed to settle for 2 min and 500 µl of the supernatant was transferred into a PowerBead tube (in duplicate for each sample). The lysis step was modified by adding 20 µl of Lysozyme [10 mg/ml], 5 µl of RNaseA [10 mg/ml], and 25 µl of Proteinase K [10 mg/ml] and by increasing the incubation period to 20 min with two rounds of vortexing (2-3 minutes each) dispersed evenly throughout this period.

The full-length 16S rRNA gene was amplified from each sample using universal bacterial primers 27F and 1492R (Suzuki and Giovannoni, 1996) that were designed as barcoded primer sets for PacBio high-throughput sequencing (Table 2). To control for potential DNA contamination in the FSW used to create the coral crushate, the FSW was autoclaved and UV sterilized prior to use. This treated FSW was used for DNA extraction and failed to yield PCR products when run as a negative control amplification, which indicates that the FSW did not influence bacterial community identification in this study. All pipets, tips, tubes, and MilliQ water used for PCR was autoclaved and UV sterilized prior to experimentation. For each sample, a new tube of DNA polymerase enzyme mix was used to avoid contamination. PCR primers were resuspended in autoclaved and UV sterilized MilliQ water.

Table 2: Oligonucleotide primers and barcodes used in this chapter.

Primer name	Primer sequence (5' → 3')		Source/citation
27F	AGAGTTTGATCMTGGCTCAG		Suzuki *et al.,* 1995
1492R	ACCTTGTTACGACTT		Suzuki *et al.,* 1995
8F	AGAGTTTGATCCTGGCTCAG		Aebischer *et al.*, 2006
1513R	GGTTACCTTTGTTACGACTT		Aebischer *et al.*, 2006
Barcode name	**Forward Barcode (5' → 3')**	**Reverse Barcode (5' → 3')**	**Source/citation**
1	GCGCTCTGTGTGCAGC	CGCAGCAGACACTGTA	Guidelines for Using PacBio® Barcodes for SMRT® Sequencing 6/16/14
2	TCATGAGTCGACACTA	CGCTACACTATCTGTA	
3	TATCTATCGTATACGC	GTCTCGAGCTCTACGA	
4	ATCACACTGCATCTGA	ATCGAGTGCGCAGCTC	
5	ACGTACGCTCGTCATA	CACATAGCGACATCGC	
6	TGTGAGTCAGTACGCG	TATGCGCTGACTCTCG	
7	AGAGACACGATACTCA	TACGTGCTCATCGTGA	
8	CTGCTAGAGTCTACAG	GATCATGATCTCAGTC	
9	AGCACTCGCGTCAGTG	AGCAGTATCATGTCGT	
10	TCATGCACGTCTCGCT	CACATCTGTGCTGTAT	
11	AGAGCATCTCTGTACT	GAGAGATATCGACTGT	
12	CGCATCGACTACGCTA	CGTCATGTGATCGAGC	
13	CGTAGCGTGCTATCAC	GTCGCACGTGTACGAC	
14	ATGCTGATGACTGCGA	CACTCTAGATATGAGT	
15	TGCGTGAGCTGTACAT	AGCGCGACAGATCAGT	
16	CGATCATCTATAGACA	GTAGTCAGAGCTAGTG	
17	CGACGTATCTGACAGT	GCTAGTACATGACAGC	
18	CACGTCACTAGAGCGA	AGTGCGTGTATGTATA	
19	TGTCGCAGCTACTAGT	GAGACGCGTCGTCACA	
20	CATACGCTGTGTAGCA	AGTGCATGCTCACGTC	
21	AGTCGCATGACTGTGT	AGCTACACGTATCGAG	
22	CAGTACTGCACGATCG	ACAGCTGTCTAGACAC	
23	GTGCTGAGCATCAGAC	TGCGTACGCATGCATC	
24	CACTGATCGATATGCA	CATCGCTGCTCTGATA	

Extracted crushate DNA was amplified using PrimeStar® Max polymerase mix (Takara Bio USA, Inc.). A master mix was prepared for each sample containing the appropriate barcoded primer pair and crushate DNA. Prior to the addition of crushate DNA, 50 µl was removed and used for PCR as a negative control for to verify that the mix was not

contaminated. Each PCR reaction volume was 50 µl; 22 µl water, 1 µl [0.25 µM] forward primer, 1 µl [0.25 µM] reverse primer, 1 µl [25 ng/ µl] crushate DNA, and 25 µl polymerase mix. Each sample was amplified in sextuplicate, totaling 300 µl of amplification reactions for each coral fragment extract. The thermocycler protocol was as follows: initial denaturation at 94 ˚C for 30 seconds, denaturation at 94 ˚C for 15 seconds, annealing at 55 ˚C for 15 seconds, elongation at 72 ˚C for 30 seconds, and a final elongation at 72 ˚C for 30 seconds. The protocol was repeated for 25 cycles to assure optimal performance of the polymerase and to avoid primer and dNTP depletion, and prevent the accumulation of side-products.

16S rRNA gene sequencing and bioinformatic processing of data.

Ten µl of each of the pooled PCR amplifications were visualized on TAE (Tris-acetate-EDTA) gels solidified with 2 % agarose to confirm amplification and correct size. The remaining 290 µl was purified using the QIAquick PCR purification kit (Qiagen, Inc.) according to the manufacturer's instructions, resulting in one amplicon product per sample. Purified PCR amplicons were submitted to the Laboratory for Biotechnology and Bioanalysis facility at the School of Molecular Biosciences, Washington State University, for high-throughput sequencing with the Pacific Biosciences RS II platform. There, amplicon concentrations and fragment length were once more assessed using an Aglient BioAnalyzer. The amplicons were pooled to be of equal nucleic acid concentrations and each collection of amplicons (21 amplicons for the outbreak and 24 amplicons for the non-outbreak) went through separate library preparations, resulting in two libraries (outbreak and non-outbreak). Each of the two libraries were analyzed using eight SMRT cells. Circular

consensus sequences (CCS) were generated within the Pacific Biosciences SMRT portal software v2.3.0 with 99 % accuracy.

All sequencing data were curated using mothur (v1.38) (Schloss *et al.*, 2009) and visualized using the PRISM7 (GraphPad Software, La Jolla, CA) software. Since there is no published mothur SOP for PacBio high-throughput sequencing data, several specific features were incorporated into a previously described mothur MiSeq SOP to analyze PacBio sequence data (Kozich *et al.*, 2013). The different fastq files from the 16 SMRT cells were merged to create one "master" fastq file. The concatenated "master" fastq file was parsed so that scores of zero were interpreted as corresponding to an ambiguous base call (i.e. N) in the fastq.info command using the pacbio=T option. Since the CCS can be generated in the forward and reverse complement orientations, the checkorient option was deployed in the trim.seqs command to identify the proper orientation. Unique sequences and their frequencies were identified in each sample. Sequences were aligned against a SILVA-based reference alignment (Pruesse *et al.*, 2007) using a profile-based aligner (Schloss *et al.*, 2009) and sequences that did not align to the correct region were culled. The ends of the sequences were trimmed to ensure all sequences started and ended at the same alignment coordinates (Schloss, 2013). All unique sequences and their frequencies were identified in the remaining pool of sequences. A pre-clustering algorithm was then applied to further de-noise sequences within each sample. *De novo* chimera detection was performed on the remaining sequences using the abundance-based algorithm implemented in UCHIME (Edgar *et al.*, 2011). The sequences were then classified against the SILVA (v123) (Pruesse *et al.*, 2007) reference taxonomy with a negative Baÿesian classifier implemented within mothur (Wang *et al.*, 2007). Sequences assigned to the phylum

Cyanobacteria or organelle Chloroplast were subjected to additional analysis to ensure that chloroplast sequences were discarded and cyanobacterial sequences were retained. The sequences in question were searched against a custom database of cyanobacterial and chloroplast sequences using BLAST as previously described (Altschul *et al.*, 1990; Janouškovec *et al.*, 2012). Sequences were assigned to operational taxonomic units (OTUs) using the average neighbor clustering algorithm with a 1% distance threshold. Previous experience (Chen *et al.*, 2013) indicates that a more stringent (e.g. 1 %) dissimilarity cut-off may be needed for high-complexity data sets. A higher dissimilarity threshold (e.g. 3 % or higher) would incorrectly group the high-similarity species with one another, which leads to a reduced resolution of the community diversity especially if communities consist of similar species. OTUs containing only one (singletons) or two sequences (doubletons) were removed from the data set (Mosher *et al.*, 2014). Shannon's *H*', Shannon's equitability index ([Shannon diversity index/log (species richness)]), rank abundance, and Chao1 rarefaction measures were calculated to assess alpha diversity, evenness, and richness of the bacterial communities. R v3.2.2 was used to conduct similarity percentage (SIMPER) analysis, which identified OTUs that contributed to differences between outbreak and non-outbreak HH, HD, and DD bacterial communities. The Mann-Whitney nonparametric test was used to evaluate differences in diversity metrics, OTU abundances, and bacterial order and phyla percentages between outbreak and non-outbreak samples. To describe the dissimilarity among the samples, the distance between communities was calculated using the Yue & Clayton measure of dissimilarity (Yue and Clayton, 2005). The resulting phylogenetic tree was used to perform a weighted UniFrac, which takes into account the abundance of OTUs present within each sample, and an analysis of similarity (ANOSIM) of variance beta

diversity analysis. To visualize the distance matrix and to assess differences in bacterial community structure in a taxonomy-independent manner, non-metric multidimensional scaling (NMDS) was used to preserve the distance between samples using two dimensions. Detailed methods, including the mothur commands, are found in the appendix of this chapter.

In silico bacterial source tracking of fecal coliform origin.

It has been shown that *Bacteroides-Prevotella* group-specific 16S rRNA gene markers show high host specificity and correlate with fecal coliforms (Okabe and Shimazu, 2007). Previously described human-, pig-, and cow-specific *Bacteroides-Prevotella* 16S rRNA gene sequences were aligned with representative sequences from OTUs of the order Bacteroidales (Okabe and Shimazu, 2007). Sequences were aligned using the ClustalW program (Kumar *et al.*, 2016) and a neighbor-joining phylogenetic tree was constructed (Saitou and Nei, 1987). Bootstrap resampling analysis of 500 replicates was performed to estimate the confidence of the tree topology.

Culturing of fecal coliform and experimental infection trials.

To obtain fecal coliform isolates for experimental infection trials with *M. capitata*, 100 µl of coral crushate was plated onto enteric-selective MacConkey agar medium (Hardy Diagnostics, Santa Maria, CA). After 48 h of incubation at 37°C, bacterial colonies that displayed lactose fermentation (medium contains neutral red pH indicator) were streaked to purity and identified by amplification and sequencing of the full 16S rRNA gene with the universal primers 8F and 1513R (Table 2). The PCR product was purified using the

QIAquick PCR purification kit (Qiagen, Inc.) according to the manufacturer's instructions and sequenced with the same primers used for amplification. Forward and reverse sequences were aligned using the CAP3 sequence assembly program (Huang and Madan, 1999) and analyzed with BLAST (Altschul *et al.*, 1990). After identifying the cultured fecal coliforms, a single representative was chosen for further analysis (= *Escherichia fergusonii* strain OCN300).

To assess the fecal coliform's ability to induce disease signs in healthy *M. capitata*, experimental infection trials were conducted. Healthy *M. capitata* fragments were collected and allowed to recover in a flow-through water table at ambient temperature for at least two days prior to experimental infection trials. Infection trials employed a block design, in which all coral fragments used within an experimental block were collected from the same coral colony to control for intraspecific variability in disease susceptibility, and were conducted as previously described (Ushijima *et al.*, 2012, 2014, 2016, Beurmann *et al., in press*) with minor modifications. Each fragment was individually housed in a four-liter tank. Tanks were filled with three liters of FSW and the water temperature was maintained at 25 ˚C. *Alteromonas* sp. OCN004 was used as a negative control bacterium as previously described (Ushijima *et al.*, 2012, 2014), *Vibrio coralliilyticus* strain OCN008 was used as a positive control bacterium that induces aMWS on *M. capitata* fragments as previously described (Ushijima *et al.*, 2014), and *Pseudoalteromonas piratica* strain OCN003 was used to test if fecal coliform pre-treatment increases the infection rate of the opportunistic aMWS-inducing pathogen (Beurmann *et al.*, *in press*). For coral inoculation, overnight liquid cultures of the fecal coliform candidate were diluted 1:1000 in LB-Miller medium, grown to an OD_{600} (optical density measured at 600 nm) of 0.8 at 37 °C, and were washed once and

resuspended in autoclaved water. Overnight liquid cultures of OCN004, OCN008, and OCN003 were diluted 1:1000 in GASW broth, grown to an OD_{600} of 0.8 at 27 °C, and were washed once and resuspended in autoclaved FSW. The isolated fecal coliform, OCN004, OCN008, and OCN003 were inoculated to a final concentration of 10^8 CFU/ml of tank water. The pre-treatment with OCN300 lasted for three days, and a partial water change was conducted prior to inoculation with OCN003 or OCN004.

In silico tracking of previously described _M. capitata_ pathogens.

The full-length 16S rRNA gene sequences form the sequencing output were screened for the presence of the 16S rRNA gene sequences of the previously described *M. capitata* pathogens, *Vibrio coralliilyticus* strain OCN008 [KF042020] and *Pseudoalteromonas piratica* strain OCN003 [KF042038].

Accession numbers.

Sequences from the PacBio RSII sequencing runs were submitted to the NCBI Sequence Read Archive under accession no. SRP096080. The fecal coliform candidate's 16S rRNA gene sequence was deposited at GenBank under accession no. KY421548.

Table 3: Average dissimilarities from SIMPER analysis for bacterial communities associated to HH, HD, and DD *M. capitata* colonies during an outbreak and non-outbreak period. Analysis of similarity (ANOSIM) of variance beta diversity measures indicate if there is a statistically significant difference between bacterial communities.

OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HD_outbreak	HD_non-outbreak				
1	*Escherichia-Shigella*	3088	867	20.54	63.89	0.06	0.145
2	*Escherichia-Shigella*	1112	339	27.69			
16	*Shimia*	839	322	32.47			
3	*Nautella*	601	256	35.66			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_non-outbreak	HD_non-outbreak				
894	*Escherichia-Shigella*	606	0	14.71	35.78	0.11	0.074
109	Oligosphaeria	257	3	20.88			
1	*Escherichia-Shigella*	987	867	23.79			
2	*Escherichia-Shigella*	447	339	26.41			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_outbreak	HD_non-outbreak				
1	*Escherichia-Shigella*	3088	867	21.88	62.44	-0.01	0.478
2	*Escherichia-Shigella*	1129	339	29.67			
16	*Shimia*	920	322	35.56			
4	*Sphingomonas*	610	225	39.35			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HD_non-outbreak	DD_non-outbreak				
1	*Escherichia-Shigella*	867	158	9.36	82.43	0.99	<0.001
16	*Shimia*	322	59	12.84			
2	*Escherichia-Shigella*	339	98	16.02			
3	*Nautella*	256	41	18.86			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		DD_outbreak	HD_non-outbreak				
1	*Escherichia-Shigella*	2599	867	16.17	64.23	0.13	0.038
9	*Escherichia-Shigella*	891	2	24.46			
2	*Escherichia-Shigella*	983	339	30.47			
16	*Shimia*	762	322	34.58			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HD_outbreak	HH_non-outbreak				
1	*Escherichia-Shigella*	3088	987	17.58	64.64	0.08	0.074
2	*Escherichia-Shigella*	1112	447	23.15			
894	*Escherichia-Shigella*	0	606	28.22			
16	*Shimia*	839	326	32.52			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_outbreak	HD_outbreak				
56	Alteromonas	36	272	5.25	19.34	0.01	0.349
135	*Escherichia-Shigella*	9	146	8.30			
16	*Shimia*	920	839	10.11			
8479	*Escherichia-Shigella*	3	49	11.13			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HD_outbreak	DD_non-outbreak				
1	*Escherichia-Shigella*	3088	158	19.70	92.05	0.99	<0.001
2	*Escherichia-Shigella*	1112	98	26.52			
16	*Shimia*	839	59	31.76			
3	*Nautella*	601	41	35.53			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HD_outbreak	DD_outbreak				
9	*Escherichia-Shigella*	3	891	13.64	27.52	0.03	0.221
1	*Escherichia-Shigella*	3088	2599	21.16			
2	*Escherichia-Shigella*	1112	983	23.14			
135	*Escherichia-Shigella*	146	31	24.90			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_outbreak	HH_non-outbreak				
1	*Escherichia-Shigella*	3088	987	18.90	62.40	0.14	0.038
2	*Escherichia-Shigella*	1129	447	25.03			
894	*Escherichia-Shigella*	0	606	30.48			
16	*Shimia*	920	326	35.83			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_non-outbreak	DD_non-outbreak				
1	*Escherichia-Shigella*	987	158	9.37	82.36	0.86	<0.001
894	*Escherichia-Shigella*	606	0	16.22			
2	*Escherichia-Shigella*	447	98	20.16			
16	*Shimia*	326	59	23.18			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		DD_outbreak	HH_non-outbreak				
1	*Escherichia-Shigella*	2599	987	13.72	64.44	0.05	0.167
9	*Escherichia-Shigella*	891	3	21.27			
894	*Escherichia-Shigella*	0	606	26.43			
2	*Escherichia-Shigella*	983	447	30.99			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_outbreak	DD_non-outbreak				
1	*Escherichia-Shigella*	3088	158	20.80	90.94	0.99	0.001
2	*Escherichia-Shigella*	1129	98	28.12			
16	*Shimia*	920	59	34.24			
3	*Nautella*	622	41	38.36			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		HH_outbreak	DD_outbreak				
9	*Escherichia-Shigella*	14	891	14.16	26.94	0.10	0.092
1	*Escherichia-Shigella*	3088	2599	22.06			
16	*Shimia*	920	762	24.61			
2	*Escherichia-Shigella*	1129	983	26.97			
OTU	SILVA classification	Average abundance		Accumulated Contribution (%)	Average dissimilarity (%)	R-value	P-value
		DD_outbreak	DD_non-outbreak				
1	*Escherichia-Shigella*	2599	158	17.27	88.84	0.75	0.003
2	*Escherichia-Shigella*	983	98	23.53			
9	*Escherichia-Shigella*	891	84	29.24			
16	*Shimia*	762	59	34.21			

APPENDIX

Mothur commands used in this analysis

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processors=4)

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count.seqs(name=all-ROI.trim.names, group=all-ROI.groups, processors=4)

align.seqs(fasta=all-ROI.trim.unique.fasta, flip=t, reference=silva.nr_v123.align, processors=4)

summary.seqs(fasta=all-ROI.trim.unique.align, count=all-ROI.trim.count_table, processors=4)

screen.seqs(fasta=all-ROI.trim.unique.align, count=all-ROI.trim.count_table, summary=all-
ROI.trim.unique.summary, start=1046, end=43116, processors=4)

filter.seqs(fasta=all-ROI.trim.unique.good.align, vertical=T, trump=., processors=4)

unique.seqs(fasta=all-ROI.trim.unique.good.filter.fasta, count=all-ROI.trim.good.count_table)

pre.cluster(fasta=all-ROI.trim.unique.good.filter.unique.fasta, count=all-
ROI.trim.unique.good.filter.count_table, processors=4)

chimera.uchime(fasta=all-ROI.trim.unique.good.filter.unique.precluster.fasta, count=all-
ROI.trim.unique.good.filter.unique.precluster.count_table, dereplicate=t, processors=4)

remove.seqs(fasta=all-ROI.trim.unique.good.filter.unique.precluster.fasta, accnos=all-
ROI.trim.unique.good.filter.unique.precluster.denovo.uchime.accnos)

classify.seqs(fasta=all-ROI.trim.unique.good.filter.unique.precluster.pick.fasta, count=all-
ROI.trim.unique.good.filter.unique.precluster.denovo.uchime.pick.count_table,
reference=silva.nr_v123.align, taxonomy=silva.nr_v123.tax, cutoff=60, processors=4)

dist.seqs(fasta=all-ROI.trim.unique.good.filter.unique.precluster.pick.fasta, processors=4,
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ROI.trim.unique.good.filter.unique.precluster.denovo.uchime.pick.count_table, cutoff=0.05)

make.shared(list=all-
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ROI.trim.unique.good.filter.unique.precluster.denovo.uchime.pick.count_table, label=0.01)

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summary.single(shared=all-
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, calc=nseqs-coverage-sobs-shannon-simpson-shannoneven-simpsoneven-boneh, subsample=12000,
iters=10000)

rarefaction.single(shared=all-
ROI.trim.unique.good.filter.unique.precluster.pick.phylip.an.unique_list.0.01.filter.shared,
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classify.otu(list=all-
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ROI.trim.unique.good.filter.unique.precluster.pick.nr_v123.wang.taxonomy, count=all-
ROI.trim.unique.good.filter.unique.precluster.denovo.uchime.pick.count_table, label=0.01)
```

```
tree.shared(shared=all-
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calc=thetayc)

unifrac.weighted(tree=all-
ROI.trim.unique.good.filter.unique.precluster.pick.phylip.an.unique_list.0.01.filter.thetayc.0.01
.ave.tre, group=all-ROI.design.txt, random=T, groups=all)

pcoa(phylip=all-
ROI.trim.unique.good.filter.unique.precluster.pick.phylip.an.unique_list.0.01.filter.thetayc.0.01
.lt.ave.dist)

nmds(phylip=all-
ROI.trim.unique.good.filter.unique.precluster.pick.phylip.an.unique_list.0.01.filter.thetayc.0.01
.lt.ave.dist)

anosim(phylip=all-
ROI.trim.unique.good.filter.unique.precluster.pick.phylip.an.unique_list.0.01.filter.thetayc.0.01
.lt.ave.dist, design=all-ROI.design.txt)
```

CHAPTER 3: ASSESSMENT OF DISEASE LESION REMOVAL AS A METHOD TO CONTROL CHRONIC *MONTIPORA* WHITE SYNDROME[1]

INTRODUCTION

Coral reefs are of great importance to human societies and the myriad reef-dwelling organisms. Reefs protect coastlines from the damaging effects of wave action (Sheppard *et al.*, 2005), provide habitats for marine organisms (Friedlander *et al.*, 2003), and harbor natural resources, such as food and sources of secondary metabolites that can serve as lead compounds for drug discovery (Belarbi, 2003; Bellwood *et al.*, 2004). Corals, like all other organisms, are susceptible to disease, and the declining health of reefs has led to disease outbreaks, incidences of which have increased worldwide (Goldberg and Wilkinson, 2004b; Bourne, 2005b; Miller and Williams, 2006). Environmental stressors, including elevated seawater temperatures, nutrient input from runoff, and sedimentation, exacerbate the declining health of corals (Harvell *et al.*, 2007; Dalton *et al.*, 2010; Haapkylä *et al.*, 2011). The tissue-loss disease *Montipora* white syndrome has impacted the population of *Montipora capitata* in Kāneʻohe Bay, Hawaiʻi over the last decade (Aeby *et al.*, 2010). Two types of tissue-loss disease have been documented: a progressive infection with diffuse tissue loss termed chronic *Montipora* white syndrome (cMWS) and a comparatively faster manifestation termed acute *Montipora* white syndrome (aMWS) (Ushijima *et al.*, 2012). Colonies exhibiting cMWS infections are observed at constant levels throughout the year (Aeby *et al.*, 2010), while aMWS infections are seasonal and occur at outbreak levels in the

[1] Beurmann *et al.* 2017. *Dis. Aquat. Org.* doi: 10.3354/dao03088

cold, rainy winter months (Aeby *et al.*, 2010). Due to the slow progression of cMWS, colonies exhibiting these lesions can survive with this disease for several months to years (Aeby *et al.*, 2010). In contrast, aMWS can lead to complete colony mortality within a few weeks (Aeby *et al.*, 2016). Field and laboratory observations suggest that corals exhibiting cMWS can switch to the acute disease manifestation, which can result in colony death (Work *et al.*, 2012). Colonies with aMWS have also been observed to revert to cMWS, but the mechanism of switching in either direction remains unknown. The widespread mortality of *M. capitata* colonies in Kāneʻohe Bay and the ability of cMWS lesions to suddenly switch to the acute manifestation of this disease stress the importance of developing a method that reduces the abundance of cMWS-afflicted *M. capitata* colonies, consequently reducing morbidity (defined as infection resulting in partial death of the colony) from disease and decreasing the risk of future aMWS outbreaks (Aeby *et al.*, 2016).

Lesion removal is a common form of medical intervention to cure some diseases affecting both vertebrates and invertebrates. Similar treatment methods employing lesion removal have proven successful for mitigating the damage inflicted by certain coral diseases (Hudson, 2000; Dalton *et al.*, 2010; Williams, 2013; Aeby *et al.*, 2015). Removing pathogen-afflicted areas of tissue by suction and covering the affected area with modeling clay was 70 % effective in controlling black band disease on affected *Oscillatoria membrancea* in the Florida Keys (Hudson, 2000). Application of a double band of marine epoxy mixed with chlorine powder to the black band disease front significantly reduced *Montipora capitata* colony mortality by 30 % compared to non-treated colonies in Kauaʻi, Hawaiʻi (Aeby *et al.*, 2015). Another study revealed that removing the disease front of a tissue loss disease affecting *Turbinaria* colonies in Australia effectively halted disease

progression in 80% of the colonies (Dalton *et al.*, 2010). In addition, removal of growth anomalies on branching acroporids in the central Pacific (Northern Line Islands) resulted in 90 % of colonies remaining disease free for nine months post-treatment (Williams, 2013). Because corals are efficient at healing injuries (i.e. re-growth of tissue) (Henry and Hart, 2005; Work and Aeby, 2010), the burden of healing wounds created by the removal of disease lesions should not have long-term impacts on the coral colonies. Although potentially confounding evidence has been reported that wounds can increase susceptibility to some diseases (Aeby and Santavy, 2006; Page and Willis, 2008), the above-mentioned treatments remain a potentially important means of containing some types of disease outbreaks in the short term. The increasingly numerous records of successful disease treatments will aid resource managers in addressing the growing threat of coral disease outbreaks.

While morbidity- and mortality-reducing techniques have been developed for many plant and animal species (Nandakumar *et al.*, 2001; Warrell *et al.*, 2008), only a few are available for corals. Therefore, our objective was to test lesion removal as a method of disease treatment to reduce morbidity or mortality from tissue loss associated with cMWS lesions.

RESULTS

Lesion removal stopped tissue loss in the treatment area

Due to environmental and anthropogenic factors (i.e. reef damage from boat strikes and reef collapse due to storms), the initial sample size of ten for each group was reduced

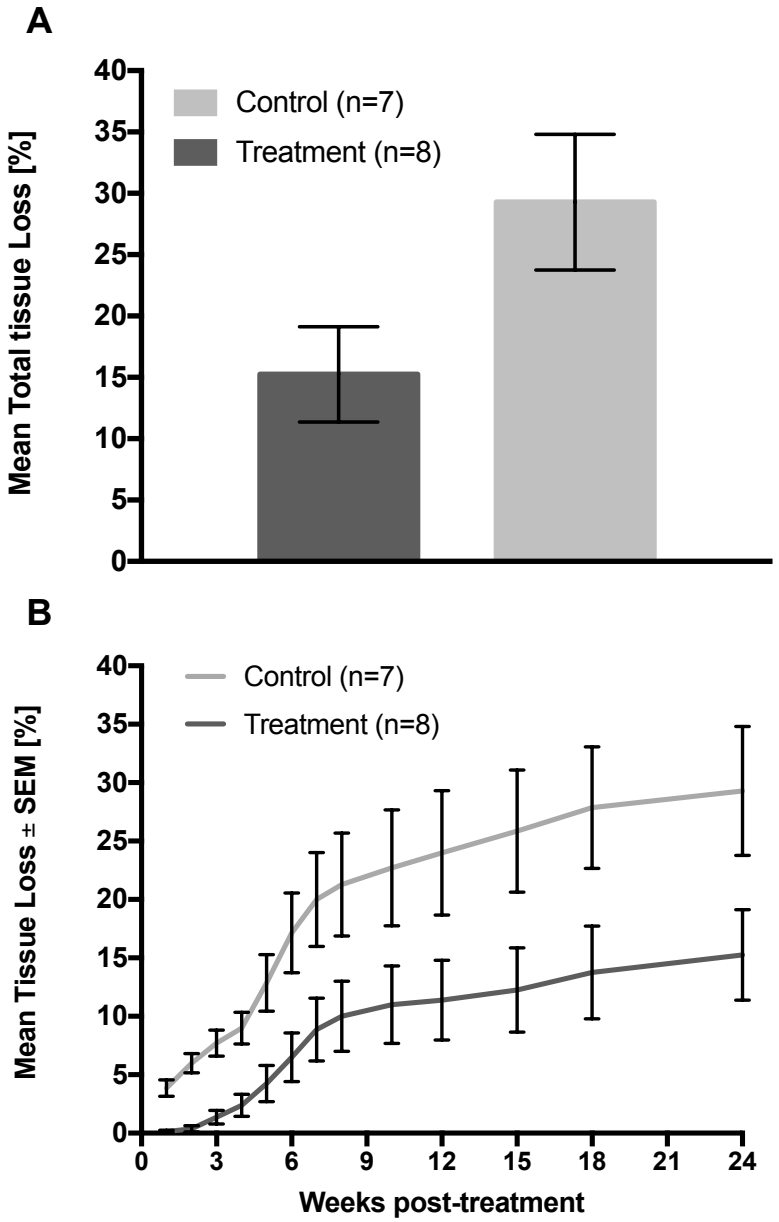

Fig. 11: Tissue loss of control and treatment colonies. (A) Total tissue loss from *M. capitata* colonies affected by cMWS. Tissue loss is presented as the mean ± standard error of the mean (SEM) after 24 weeks post-treatment from control colonies and those treated via lesion removal. (B) Mean percent tissue loss ± SEM on *M. capitata* treatment colonies (those with lesions removed, n = 8) and control colonies (cMWS lesions left in place, n = 7) throughout the study period of 24 weeks.

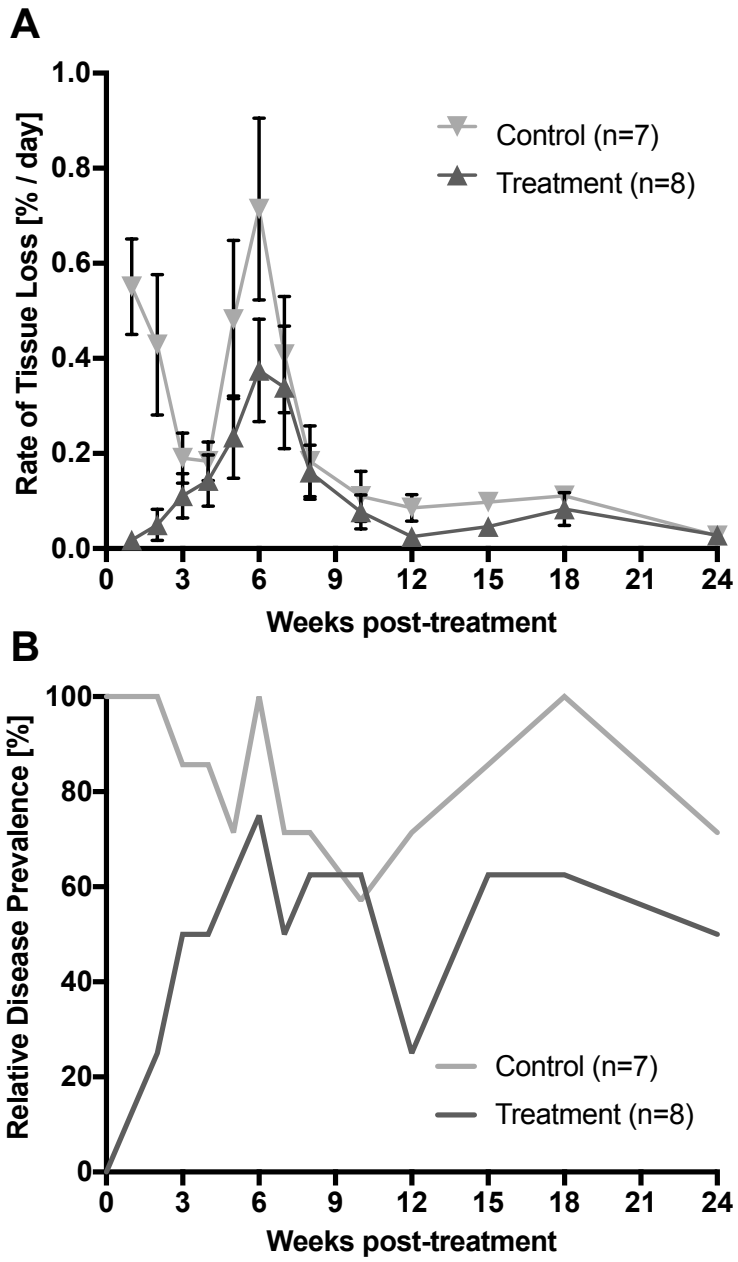

Fig. 12: Disease progression of treatment and control colonies. (A) The rate of tissue loss, calculated as the percent tissue loss per day across each sampling interval, and (B) the relative disease prevalence of active cMWS lesions through time on treated (those with lesions removed) and control (cMWS lesions left in place) *M. capitata* colonies during the study period of 24 weeks.

to seven for the control and eight for the treatment colonies. Within the treatment group, no further tissue loss occurred in the area of lesion removal. All wounds created from treatment healed and were grossly covered with tissue within five weeks post-removal. In contrast, lesions present within the control group continued to progress over the course of the study. Lesion removal did not prevent re-infection, and the disease reoccurred in some treatment colonies as early as seven days post-treatment and continued through time. After the 24 weeks, all of the control colonies (n = 7) and seven of the eight treatment colonies exhibited new lesions, and the relative percentages of disease prevalence displayed similar trajectories between treatment and control colonies (Fig. 12B). Re-infections did not occur around the treatment margins but only on other areas of the colony.

Lesion removal reduced overall morbidity in treatment colonies

Within the 24-week observation period, lesion removal resulted in reduced morbidity in treatment colonies. An assessment of the initial and final percentages of tissue loss showed that treatment colonies lost almost half the amount of tissue that was lost by control colonies, a mean total of 48 % less tissue (Fig. 11A; treatment colonies 15.25 % ± SEM 3.89 % compared to control colonies 29.29 % ± SEM 5.53 %), but this difference was not statistically significant (Mann-Whitney, U = 13.5, n_C = 7, n_T = 8, p = 0.101). Comparison of the mean percent tissue loss at each sampling interval displayed a statistically significant decrease in tissue loss by treatment colonies (1.17 % ± SEM 0.47 %) compared to control colonies (2.25 % ± SEM 0.63 %) over the course of the experiment without accounting for time (Fig. 11B; rmMANOVA: F(13, 1) = 1239.1, p = 0.022). Once time was accounted for, a

statistically significant interaction effect between the percentage of tissue loss and time was also observed (rmMANOVA: $F(13, 1) = 1191.3$, $p = 0.023$).

Lesion removal significantly reduced the rate of tissue loss

The average rate of tissue loss through the duration of the study, calculated as the percent tissue loss per day in Figure 12A, was also significantly lower on the treatment colonies (0.13 % ± SEM 0.04 %) compared to the control colonies (0.27 % ± SEM 0.08 %; rmMANOVA: $F(1, 13) = 8.3$, $p = 0.013$). The rate of tissue loss was calculated for each individual sampling interval, rather than over the entire experimental time course, and a comparison of these rates showed a significant difference along a fine time scale (rmMANOVA: $F(1, 12) = 20$, $p = 0.049$). By comparing daily rates of tissue loss between sampling intervals, a spike in the rate of tissue loss was observed in both groups with a maximum in week six. Interestingly, one of the control colonies exhibited the signs of a switch from a chronic to an acute lesion in the last sampling interval, but no acute tissue loss lesions were observed on the treatment colonies. Despite the increased rate of tissue loss from aMWS infections, the late timing of the switch in disease state did not alter the average rates of tissue loss calculated.

DISCUSSION

In this study, we applied a lesion removal technique to cMWS-infected *M. capitata* colonies to assess its viability as a treatment measure. Lesion removal stopped tissue loss at the initial site of infection and all wounded colonies healed within five weeks of treatment. However, re-infection was observed and new lesions appeared on other areas of

every control colony and all but one treatment colony (Fig. 12B). Lesion removal was only performed once in this study, so re-infection resulted in continued tissue loss on treated colonies. Tissue loss resulting from new lesions was likely the reason why the difference in total percent tissue loss did not reach statistical significance. But even with re-infection of treatment colonies over the 24-week observation period, control colonies lost nearly twice as much tissue as treated colonies. Despite the likelihood of reinfection, the removal of disease lesions from treatment colonies significantly decreased the rate of tissue loss from cMWS on colonies. This suggests that a static endpoint picture of tissue loss is insufficient to describe the dynamics of cMWS infection and spread and the effect of treatment. *Montipora capitata* grows less than 2.5 cm/year in Hawai'i (Jokiel, 1978), and so tissue loss from disease may require substantial recovery time. These results differ from previous studies in which lesion removal was successful at stopping disease progression. Lesion removal halted white syndrome on *Turbinaria mesenterina* (Dalton et al., 2010) and growth anomalies on *Acropora acuminata* (Dalton *et al.*, 2010; Williams, 2013). The etiologies and ecologies of diseases differ, which affects the efficiency of any treatment method. The more that is understood about disease ecology, the higher the likelihood of developing an appropriate method of control. Due to the declining state of many coral reefs, a treatment method that reduces morbidity in affected colonies may be warranted.

Previous research has proposed that disruption of the coral animal through mechanical injury increases susceptibility to some diseases (Aeby and Santavy, 2006; Page and Willis, 2008) suggesting that corals are either more susceptible because they have an open wound that can subsequently become infected or that some systemic change takes place to weaken the entire organism and make it generally more susceptible to disease. In

our study, although treated colonies were re-infected with cMWS within a few weeks post-lesion removal, new infections never occurred at the removal site, indicating that tissue disruption was not required for cMWS initiation. In addition, the development of new lesions was similar between the two groups, suggesting that the processes affecting cMWS pathogenesis continued throughout the experiment regardless of coral wounding.

It is possible that a fundamental difference exists between the fresh wounds and uncompromised areas on treated colonies because all new cMWS lesions occurred on uncompromised areas of the colonies. Van de Water *et al.* (2015) examined the regulation of the coral immune response during wound healing in *Acropora aspera*. They showed that various components of the innate immune system were upregulated and suggested that an initiated immune response may prevent infection at wound sites. It is not known whether *M. capitata* responds to wounding in a similar manner. However, the occurrence of new lesions on uncompromised areas of *M. capitata* colonies is more consistent with an existing interaction that sporadically activates rather than opportunistic infections occurring at injury sites.

The data presented here indicate that disease lesions alternated between active (recent tissue loss) and inactive (no recent tissue loss) states in both groups as has been previously reported for this disease (Aeby *et al.*, 2010; Work *et al.*, 2012). In our study, disease prevalence and the rates of tissue loss showed similar temporal patterns for both groups (treatment and control) with spikes in prevalence and lesion rate occurring in weeks 3-8 (Fig. 12AB). After these spikes, the rate of tissue loss returned to a consistently lower level but prevalence continued to vacillate. The similar temporal pattern of infection among corals suggests that there may be an undiscovered environmental component

affecting cMWS processes in *M. capitata*, which could have caused these spikes. Without the temporal resolution afforded by weekly sampling, this short-term increase in infection would have been missed.

In previous work, decreases in morbidity and mortality were recorded following treatment of different types of disease lesions from various coral species (Hudson, 2000; Dalton *et al.*, 2010; Williams, 2013; Aeby *et al.*, 2015). Each of these studies reported benefits to coral survival as a result of treatment and should be considered as potential management actions in response to disease outbreaks. The differences in coral species, pathogens, and local environmental factors require that treatments be tailored to individual diseases on each coral host. For example, Williams (2013) found that growth anomaly removal was a successful treatment for *Acropora acuminata* but not for *Montipora efflorescens* located on the same reef under similar environmental conditions. Although somewhat time consuming, lesion removal would be effective in controlling disease in closed systems such as coral nurseries, at the onset of a disease outbreak, on reefs with a low incidence of cMWS, or for treating colonies that are of the most value to the reef (Raymundo *et al.*, 2008). While lesion removal has proven beneficial for cMWS-infected colonies, management actions that address the overarching drivers of coral disease (e. g. nutrient runoff from human and animal waste, overfishing and rising surface seawater temperatures) are required to maintain the long-term viability of coral reefs (Bruckner, 2002). The complexity of a coral's response to disease, the efficacy of lesion treatment, and the response of different pathogens to treatment are all areas that require further study to refine treatment options. Equally important to the initial disease response, however, is the

follow-up research required to identify and understand underlying factors that trigger disease outbreaks.

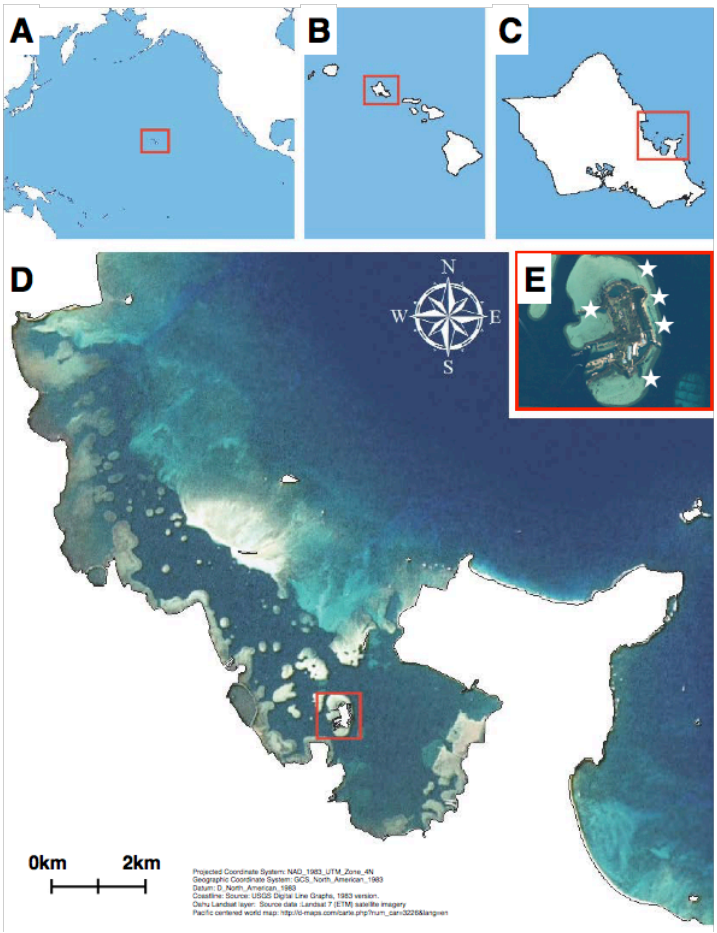

Fig. 13: Location of (A) Hawai'i, (B) O'ahu, (C) Kāne'ohe Bay, (D) Moku o Lo'e. (E) The study focused on coral colonies at five sites (stars) on the shallow fringing reefs of Moku o Lo'e.

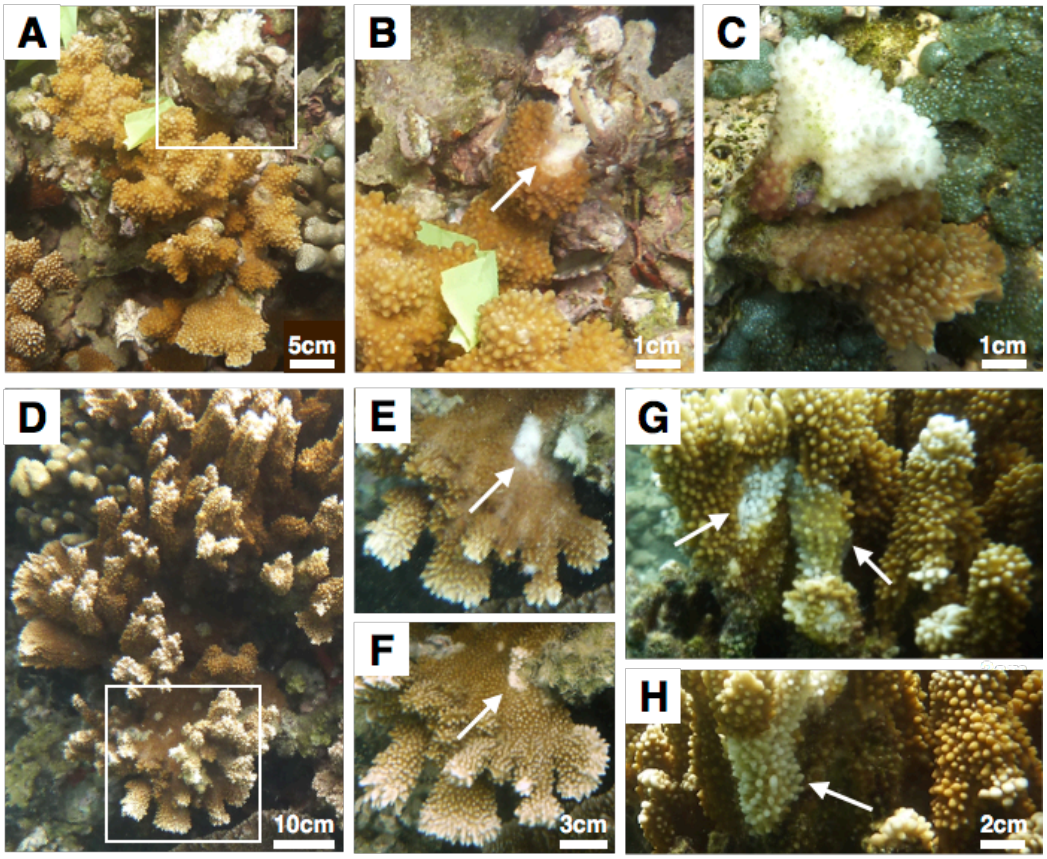

Fig. 14: Lesion removal in *M. capitata* colonies affected by cMWS. (A-C) *M. capitata* colony showing positive response to lesion removal. (A) May 2014, cMWS lesion present (box). (B) Wound left after removal of lesion (C) in May 2014 (arrow). (D-H) *M. capitata* showing development of new cMWS lesions after treatment. (D) May 2014, cMWS lesion present (box). (E) Wound left after lesion removal in May 2014 (arrow). (F) Re-growth of healthy tissue over wound within 5 weeks post-lesion removal (arrow). (G+H) Development of new cMWS lesions on other areas of colony (arrows).

MATERIALS AND METHODS

Study sites

Experimental manipulations were conducted in Kāneʻohe Bay, Oʻahu, Hawaiʻi, an intricate estuarine system with a barrier coral reef and various patch and fringing reefs (Hunter and Evans, 1995). The five study sites were separated by a minimum of 150 m and were located on the South, East, and West regions of the fringing reef surrounding the island of Moku o Loʻe in south Kāneʻohe Bay (Fig. 13). These fringing reefs have relatively low coral diversity and are dominated by two coral species, namely *Porites compressa* and *Montipora capitata* (Aeby *et al.*, 2010). This study focused on coral communities on the shallow (<5 m) fringing reefs.

Experimental removal of cMWS lesions

Chronic *Montipora* white syndrome (cMWS) is a common disease of *M. capitata* in Kāneʻohe Bay, and cMWS lesions are observed persistently (average prevalence ranged from 0.02% to 0.87%) in coral populations with no seasonal variation (Aeby *et al.*, 2010). Colonies of *M. capitata* were manipulated in the field under Special Activities Permits SAP#2013–47 and SAP#2015–17 granted by the State of Hawaiʻi, Department of Land and Natural Resources, Division of Aquatic Resources. In May 2014, a total of twenty *M. capitata* colonies with cMWS were chosen haphazardly: ten as treatment colonies and ten corresponding control colonies with comparable size lesion(s) located near each treatment colony (four/site). To facilitate complete lesion removal with minimal damage to the parent colony, only colonies displaying cMWS lesions of 1 to 5 cm^2 were chosen. All colonies were tagged, photographed, and their positions recorded using GPS. The disease

lesions, as well of roughly 2 cm of adjacent healthy tissue to ensure complete lesion removal, were removed from the treatment colonies using bone shears. *M. capitata* has a soft skeleton, and sections can be removed easily with minimal harm to the colony. All extracted diseased fragments were immediately quarantined in Ziploc bags at depth following removal and were sterilized with a 5 % sodium hypochlorite solution upon return to the laboratory. The control colonies were left untreated. For 24 weeks, all colonies were examined weekly for progressive tissue loss or the development of new lesions, and all occurrences were photo-documented (Fig. 14). The complex structure of the *M. capitata* colonies precluded the use of digital measurements to estimate rates of tissue loss. Hence, *in situ* observations on the proportion of the colony that was healthy, diseased, or dead was recorded during each survey period.

Data analyses

Due to the ordinal nature of the single dependent variable (tissue loss), and the relatively small and unequal sample sizes (two levels: treated and control colonies), a non-parametric Mann-Whitney *U* test was used to assess the effect of treatment on the total loss of healthy tissue from infected colonies. The relative prevalence of disease represents the proportion of colonies (controls or treatments) that showed active cMWS lesions at the time of survey. A repeated measures MANOVA was used to compare the rates of tissue loss and the percentages of tissue loss through time between the control and treatment colonies. The rate of tissue loss was assessed for each colony by comparing the percentage of diseased tissue at the beginning and end of each individual sampling period and expressing the rate as a daily percentage of tissue lost. These data consist of repeated

measures that are dependent levels of one independent variable. Though the residuals follow a pattern that satisfies a normal distribution when analyzed on a Q-Q plot, compound symmetry was not met (Mauchly's sphericity: $\chi^2 = 490.56$, df = 90, $p < 0.01$), so a repeated measures MANOVA test was utilized. Statistical analyses were conducted using the PRISM7 (GraphPad Software, La Jolla, CA) and JMP12 (SAS Institute Inc., Cary, NC) software packages.

CHAPTER 4: EXPOSURE TO *PSEUDOALTEROMONAS PIRATICA* RESULTS IN THE SWITCH FROM CHRONIC TO ACUTE LESIONS IN THE CORAL *MONTIPORA CAPITATA*

INTRODUCTION

Primary infection refers to the first time an organism is exposed to a pathogen, whereas a secondary infection is elicited by a pathogen that is succeeding a primary infection (Aly, 1996). Opportunistic pathogens fall within the category of secondary pathogens and are ordinarily in contact with the host, but cause disease only when the host's immune system is compromised or during dysbiosis of the normal commensals (Peterson, 1996). Superinfections are another form of secondary infection that typically occur immediately after or on top of an earlier infection (Middleton *et al.*, 1993). In contrast, co-infections are commonly caused when a host is simultaneously infected by multiple pathogens (Abruzzi and Fried, 2011). Generally, secondary infections and co-infections have an increased morbidity and lead to mortality more frequently than primary infections with the same pathogens (Pittet *et al.*, 1993).

The coral diseases that have been described to date with known etiologies are caused by either a single primary pathogenic strain or as co-infections by bacterial consortia. Studies on white pox disease in *Acropora palmata*, bleaching of *Oculina patagonica*, and white syndrome in *Pocillopora damicornis* demonstrated that *Serratia marcescens* (Sutherland and Ritchie, 2004), *Vibrio shiloi* (Kushmaro *et al.*, 1997), and *Vibrio coralliilyticus* (Ben-Haim and Rosenberg, 2002) acted as single causative agents, respectively. However, coral infections with black band disease lesions (Cooney *et al.*,

2002; Frias-Lopez *et al.*, 2003; Aeby *et al.*, 2015) have been shown to be due to co-infections by an array of different bacterial strains. Based on the differing modes of infection in humans, and the results of the above studies that have shown that primary pathogens and co-infections by consortia are possible on coral, it is possible that secondary infection may be an additional route to coral disease.

Koch's postulates of disease causation are a set of guidelines that are commonly used to infer the causality of a microorganism as a disease agent (Koch, 1876): (I) The microorganism must be present in all organisms that have the disease and should be absent in healthy organisms, (II) the microorganism should be isolated from a diseased organism and can be grown in pure culture, (III) the cultured microorganism should cause disease signs if inoculated into healthy hosts, and (IV) same microorganism must be re-isolated from the inoculated, diseased hosts. These postulates have been utilized by researchers to describe causative agents of coral diseases, although few causal relationships linking pathogens to a coral disease have been successfully demonstrated to date (but see Ben-Haim and Rosenberg, 2002; Patterson *et al.*, 2002; Denner *et al.*, 2003; Sussman *et al.*, 2008; Ushijima *et al.*, 2012, 2014, 2016). While fulfillment of Koch's postulates has proven useful in organismal studies, the advent of genetic analysis prompted repurposing of these postulates for use in molecular pathogenesis research.

Using an analogous line of reasoning, studies must fulfill Koch's molecular postulates to assess the role of a gene in a pathogen's virulence (Falkow, 1988). Like Koch's postulates, for a gene to be considered a virulence factor, mutations abrogating gene function lead to decreased virulence of the pathogen. Complementation of the mutation via reintroduction of the gene to restore function results in a concurrent return to wild type

virulence levels. To date, only a handful of genes have been implicated as virulence determinants in coral pathogens. A toxin and a superoxide dismutase have been proposed to be virulence factors produced by the pathogen *Vibrio shiloi*, which causes coral bleaching of *Oculina patagonica* (Banin *et al.*, 2001, 2003). Another study demonstrated that a metalloprotease produced by *V. coralliilyticus* strains BAA-450 and P1-4 caused tissue lesions in *Pocillopora damicornis* and *Acropora millepora,* respectively (Ben-Haim *et al.*, 2003; Sussman *et al.*, 2009). In a recent study, mutation of a toxin regulator (*toxR*) and a gene of the type IV pilus operon (*mshA*) in *V. coralliilyticus* strain OCN008 and OCN014 significantly reduced infection rates in *M. capitata* and *Acropora cytherea*, respectively (Ushijima *et al.*, 2016). While such studies on the molecular basis of pathogenesis have been conducted extensively in human pathogens, similar work is almost entirely lacking from coral disease research.

Montipora white syndrome (MWS) is a tissue loss disease that has damaged populations of *Montipora capitata,* a major reef-building coral in Kāneʻohe Bay, Hawaiʻi (Aeby *et al.*, 2010). Two types of MWS have been documented; a chronic, progressive infection that displays diffuse tissue loss termed chronic MWS (cMWS) (Ushijima *et al.*, 2012), and a comparatively faster progressive tissue loss disease termed acute MWS (aMWS) (Ushijima *et al.*, 2014; Aeby *et al.*, 2016). *Montipora capitata* colonies exhibiting cMWS infections are observed at consistent levels throughout the year (Aeby *et al.*, 2010), while aMWS infections occur sporadically (Aeby *et al.*, 2016). Aeby *et al.* (2016) found 338 and 1,232 aMWS-affected colonies in the 2010 and 2012 outbreaks, respectively. Colonies with cMWS lesions can survive with this infection for months to years because of the comparably slower rate of tissue loss (Aeby *et al.*, 2010). In contrast, aMWS-infected

colonies can die within days to weeks during disease outbreaks (Aeby *et al.*, 2016). Disease transmission was observed between neighboring *M. capitata* colonies in the field, and cMWS-infected colonies were also observed to spontaneously switch to aMWS, which commonly led to complete colony mortality (Aeby *et al.*, 2016). While this switch from chronic to acute MWS lesions has been observed in laboratory infection trials and on coral colonies in the field (Work *et al.*, 2012; Ushijima *et al.*, 2012; Beurmann *et al.*, 2016), the mechanism underlying the change in disease state remains unknown. To date, two pathogenic *Vibrio* species have been shown to act as causative agents of cMWS and aMWS in *M. capitata*, *Vibrio owensii* strain OCN002 and *Vibrio coralliilyticus* strain OCN008, respectively (hereafter referred to as OCN002 and OCN008) (Ushijima *et al.*, 2012, 2014). During controlled laboratory infections of *M. capitata*, OCN002 caused cMWS lesions in 53% of inoculated coral fragments in an average of 28 days post-inoculation (Ushijima *et al.*, 2012), while OCN008 infected 80 to 100% of fragments resulting in aMWS lesions within four days post-inoculation (Ushijima *et al.*, 2014, 2016). The virulent nature of aMWS and the ability of cMWS lesions to switch to aMWS present a threat to the survival of *M. capitata* in Kāneʻohe Bay. To date, the majority of verified singular primary coral pathogens, and both of those causing MWS, belong to the *Vibrio* genus. We sought to isolate and characterize non-*Vibrio* etiological agents of MWS and investigate the switch from cMWS to aMWS in controlled laboratory experiments.

In this study, a novel *Pseudoalteromonas* species, *P. piratica* strain OCN003 (Beurmann *et al.*, *in press*), was isolated from diseased *M. capitata* mucus. Strains of *P. piratica* were subsequently isolated from diseased corals during an outbreak of aMWS two years later. Koch's postulates of disease causation were fulfilled for *P. piratica* strain

OCN003, which establishes it as an etiological agent of acute tissue loss in *M. capitata*. When inoculated onto coral fragments exhibiting cMWS disease signs, OCN003 induced aMWS lesions more readily than on healthy coral fragments, which suggests that OCN003 acts as a secondary pathogen of *M. capitata* causing a switch from a chronic to an acute infection. As a control to show that the switch from cMWS to aMWS was due to inoculation with wild type OCN003 and that motility is a critical component of the infection process, a non-motile Δ*fliF*::*bla* mutant of OCN003 was constructed and was found to be incapable of inducing the switch in disease state. The complementation of the non-motile mutant restored the bacterium's ability to cause the switch from cMWS to aMWS lesions, fulfilling Koch's molecular postulates of disease causation. This work describes both the first strain of *Pseudoalteromonas* that infects coral and the first coral pathogen involved in secondary infections.

RESULTS

Pseudoalteromonas piratica strain OCN003 is an etiological agent of the disease acute *Montipora* white syndrome in Kāneʻohe Bay.

Pseudoalteromonas piratica strain OCN003 was identified as part of a study designed to isolate cultivable bacteria that contribute to aMWS. In this study, 67 bacteria were cultured from the mucus of healthy *M. capitata* fragments and 205 from diseased fragments. Each was identified by 16S rRNA gene sequencing. Strains belonging to the genera *Vibrio* (65 %) and *Pseudoalteromonas* (13 %) were the most abundant bacteria cultured from diseased corals, and bacteria from the genus *Pseudoalteromonas* were not isolated from healthy coral (Fig. 15). Despite the small sample size, the prevalence of

Pseudoalteromonas spp. in the mucus of diseased but not healthy coral suggested that these organisms may either be pathogens or opportunistic colonizers. Consequently, one of the 26 *Pseudoalteromonas* strains isolated from diseased coral was characterized as the novel bacterial species *Pseudoalteromonas piratica* strain OCN003 (Beurmann *et al.*, *in press*; hereafter OCN003) and assessed for virulence against *M. capitata*. Of the healthy fragments of *M. capitata* exposed to OCN003, 33% developed acute tissue loss lesions in an average of 22 days post-inoculation (McNemar's test, $n = 24$, $p = 0.01$; Fig. 16). The lesions appeared similar to aMWS lesions observed in the field and previous laboratory experiments (Ushijima *et al.*, 2014; Aeby *et al.*, 2016). As controls, *M. capitata* fragments were exposed to filtered seawater (FSW) or the negative-control bacterium, *Alteromonas* sp. strain OCN004 (Ushijima *et al.*, 2012), neither of which induced disease ($n = 24$ per treatment). These results indicate that OCN003 can act as a primary pathogen and induce aMWS infections in *M. capitata* fragments under laboratory conditions.

Prior studies defining coral pathogens as etiological agents of disease demonstrated the successful re-isolation of the pathogenic strain from experimentally infected coral fragments (Ben-Haim and Rosenberg, 2002; Ushijima *et al.*, 2012, 2014, 2016). To facilitate re-isolation and ensure that the re-isolated bacteria were derived from the laboratory stock culture, OCN003 was genetically tagged with a non-self-transmissible plasmid, pRL1383a, as previously described (Ushijima *et al.*, 2012). The infection rates between the wild type and tagged OCN003 were not significantly different (conducted simultaneously; Mantel-Cox test, $n = 20$, $p = 0.98$), and both strains induced acute tissue loss in 20 % of the fragments after an average of 19 days post-inoculation. Tagged OCN003 was re-isolated from all experimentally infected fragments that developed aMWS ($n = 20$). Collectively,

these results demonstrated that OCN003 was isolated from diseased coral, grown in pure culture, used to experimentally infect laboratory specimens, and was re-isolated from infected specimens. This fulfillment of Koch's postulates of disease causation indicates that OCN003 is an etiological agent of aMWS.

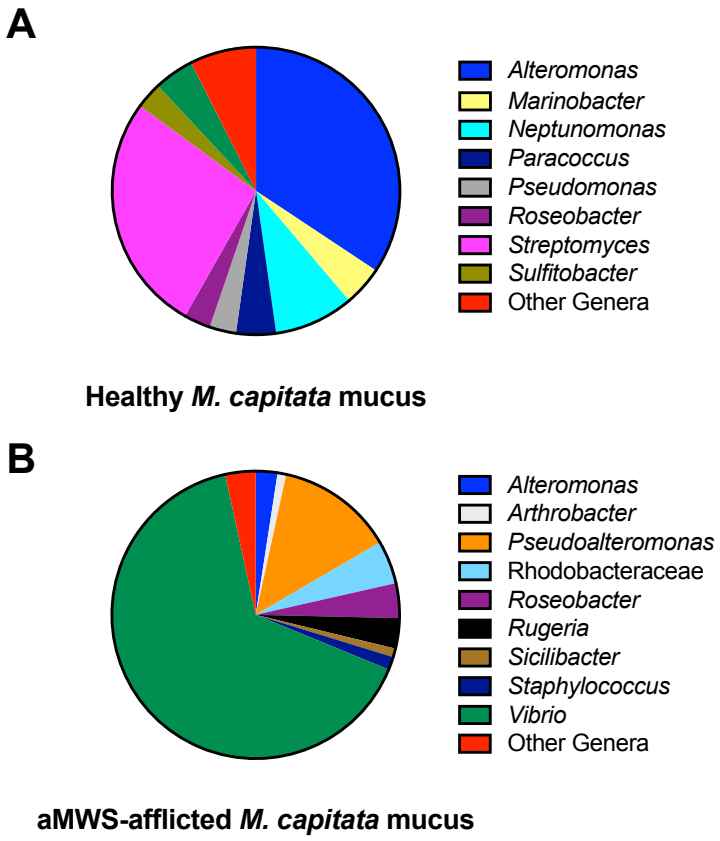

Fig. 15: Distribution of culturable bacteria identified from healthy *M. capitata* and aMWS-afflicted *M. capitata*. (A) Genera in high abundance in healthy mucus were *Alteromonas* (32 %) and *Streptomyces* (28 %); (B) while the genera *Vibrio* (65 %) and *Pseudoalteromonas* (13 %) were in high abundance in diseased (aMWS) mucus.

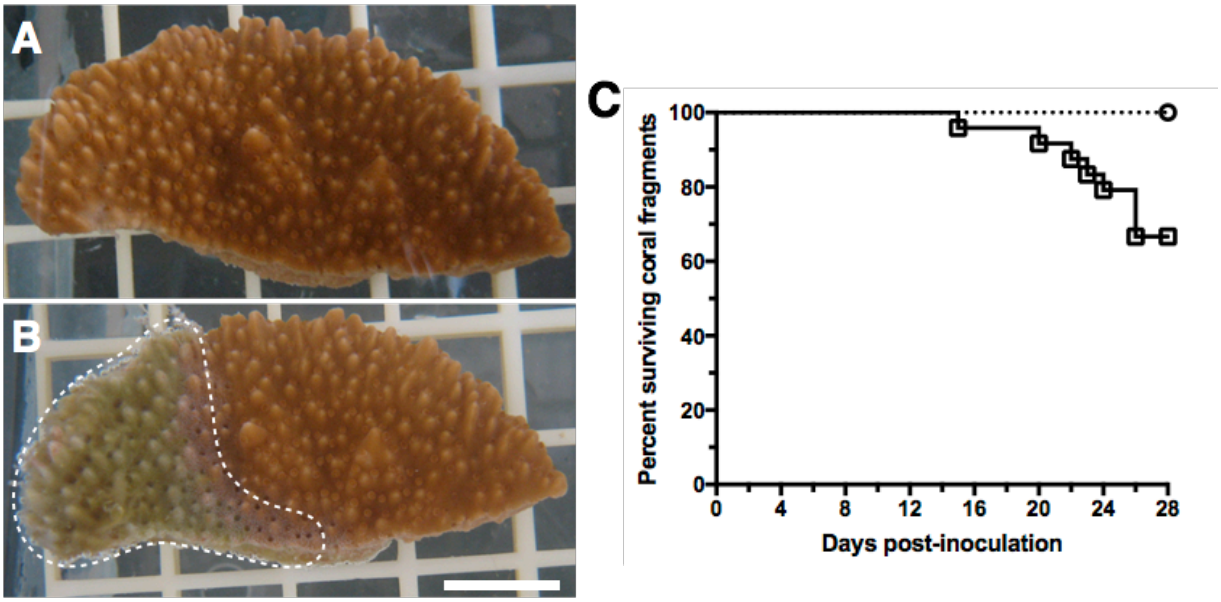

Fig. 16: OCN003 infection of *Montipora capitata*. (A) *M. capitata* before inoculation. (B) *M. capitata* 21 days post-inoculation with OCN003 displaying acute tissue loss (white dashed line). The white scale bar represents one cm. (C) Kaplan–Meier survival curve of *M. capitata* fragments exposed to OCN003 or the control bacterium, OCN004, at 25˚C. The solid line with open black squares represents fragments exposed to OCN003 (n = 24); the dotted line with open circles represents fragments exposed to OCN004 (n = 24).

P. piratica strain OCN003 acts as a secondary pathogen of *M. capitata.*

In the field, cMWS lesions on *M. capitata* colonies have been observed to develop increased progression rates that resemble aMWS lesions (Aeby *et al.*, 2016). This switch from a cMWS to an aMWS lesion could be due to many factors, one of which is the onset of a secondary infection. To investigate whether OCN003 could induce a switch from a cMWS to an aMWS lesion, *M. capitata* fragments displaying signs of cMWS were collected from the field and inoculated with OCN003. Following inoculation with OCN003, 56% of fragments with pre-existing cMWS lesions switched to aMWS lesions an average of eight days post-

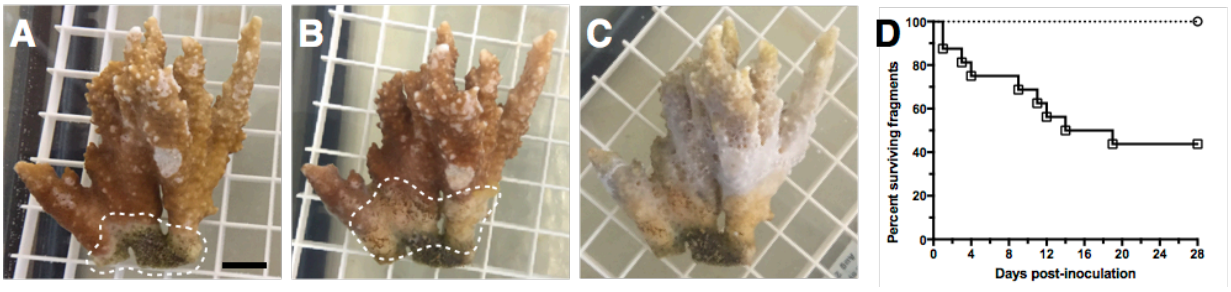

Fig. 17: OCN003 infection of *Montipora capitata* fragments with a cMWS lesion. (A) *M. capitata* with cMWS lesion (white dashed line) before inoculation. (B) *M. capitata* three days after inoculation with OCN003, showing progressing necrotic tissue (aMWS; white dashed line). (C) *M. capitata* five days after inoculation with OCN003, displaying a complete loss of healthy tissue due to aMWS. The black scale bar represents one cm. (D) Kaplan–Meier survival curve of cMWS-afflicted *M. capitata* fragments exposed to OCN003 or the control bacterium, OCN004, at 25 ˚C. The solid line with open black squares represents fragments exposed to OCN003 (n = 16); the dotted line with open circles represents fragments exposed to OCN004 (n = 16).

inoculation (McNemar's test: n = 16, p < 0.01; Fig. 17). Following the OCN003-induced switch from cMWS to aMWS in the laboratory, the newly formed aMWS lesions appeared identical to the acute tissue loss lesions described above (Fig. 18). The genetically-tagged OCN003 strain was also able to cause a switch from cMWS to aMWS lesions similarly to the wild type (Mantel-Cox test: n = 6, p = 0.92), and could be re-isolated from all *M. capitata* fragments that switched from cMWS to aMWS. The FSW or OCN004 negative controls did not induce a switch from chronic to acute lesions (n = 16 per treatment; Fig. 19). To further characterize the dynamics of lesion switching, the minimum dose of OCN003 required to induce the cMWS to aMWS switch was determined by conducting infection trials with inoculum concentrations ranging from 10^4 to 10^8 CFU/ml of tank water. After inoculation with 10^4 and 10^5 CFU of OCN003 per ml of tank water, 0% and 18% of fragments developed

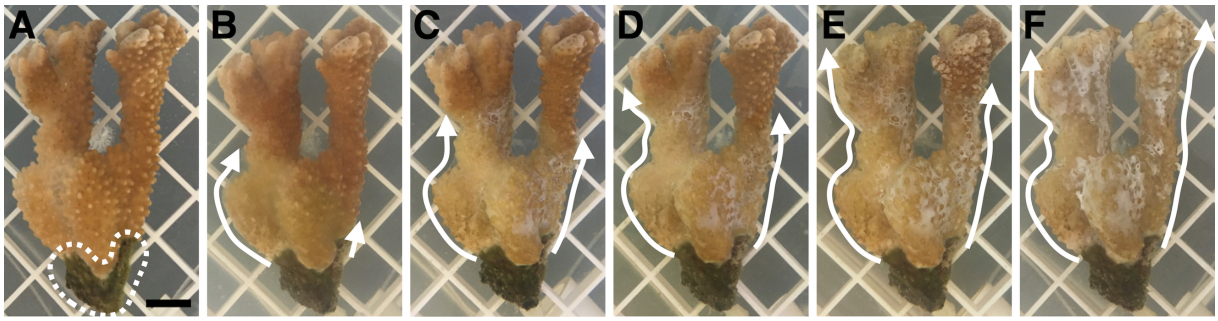

Fig. 18: OCN003 infection of cMWS-afflicted *M. capitata*. (A) *M. capitata* with cMWS lesion (white dashed line) before inoculation. *M. capitata* fragment two days (B), three days (C), four days (D), five days (E), and six days (F) post-inoculation with OCN003 displaying progressing acute tissue loss lesion (white arrows). The black scale bar represents 1 cm.

aMWS lesions, respectively. Therefore, the minimum dose required to induce the switch from cMWS to an aMWS lesion was found to be between 10^4 and 10^5 CFU/ml (n = 11). These results collectively fulfill Koch's postulates to confirm that OCN003 acts as a secondary pathogen to induce the switch from chronic to acute MWS lesions.

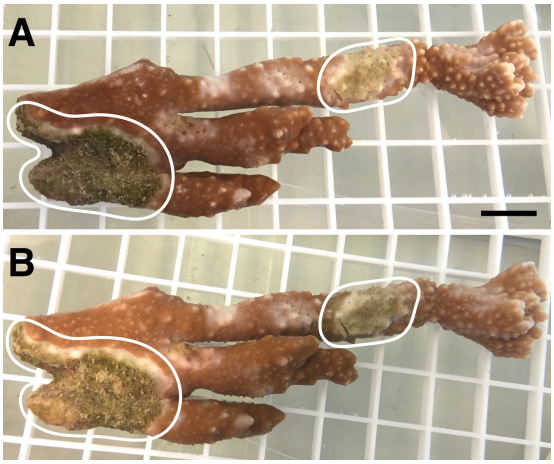

Fig. 19: Inoculation of *Montipora capitata* fragments exhibiting cMWS lesions with the control bacterium, OCN004. (A) *M. capitata* with cMWS lesions before inoculation (white line). (B) *M. capitata* 28 days after inoculation with OCN004, showing persistent cMWS lesion (white line). The black scale bar represents 1 cm.

Motility is required for OCN003 in infect as a secondary pathogen.

Secondary infection as a mode of disease causation is unprecedented in corals. Therefore, assessment of a mechanistic basis of virulence underlying the switch between MWS disease states is pertinent. Motility as a virulence determinant in induction of an acute infection from a chronic lesion, a non-motile OCN003 mutant was created. Previous studies analyzing motility as a requirement for infection have demonstrated that a *flhA* mutant in *Vibrio coralliilyticus* strain YB2 failed to form a flagellum and was incapable of infecting the coral *Pocillopora damicornis* (Meron *et al.*, 2009). In contrast, non-motile mutant strains of the human pathogen *Vibrio cholerae* were shown to remain infectious (Richardson, 1991). Rather than remove the flagellum as was previously done in *V. coralliilyticus* strain YB2 (Meron *et al.*, 2009), a mutation abolishing flagellar movement was constructed in OCN003 to preserve the cell's morphology but prevent swimming of the organism. Previous work has shown that flagella can be immunogenic (Legnani-Fajardo *et al.*, 1991; Campodónico *et al.*, 2010; Ghose *et al.*, 2016), so the introduction of two simultaneous phenotypic changes from one mutation, the lack of a flagellum and abrogation of motility, would introduce additional difficulty into the data interpretation. Therefore, the *fliF* homolog in OCN003, which encodes a flagellar motor protein, was deleted to create a non-motile mutant. Deletion of *fliF* in other bacteria results in the production of a non-functional flagellum, which abolishes bacterial motility and potentially impairs adhesion to the host (Grünenfelder *et al.*, 2003; Bigot *et al.*, 2005). The non-motile strain of OCN003 was created by replacing all but the first and last 18 nucleotides of the coding region of the *fliF* homolog in OCN003 [AIY66328] with the *bla* gene, which confers resistance to the antibiotic ampicillin. The resulting strain, OCN003 Δ*fliF*::*bla*, displayed

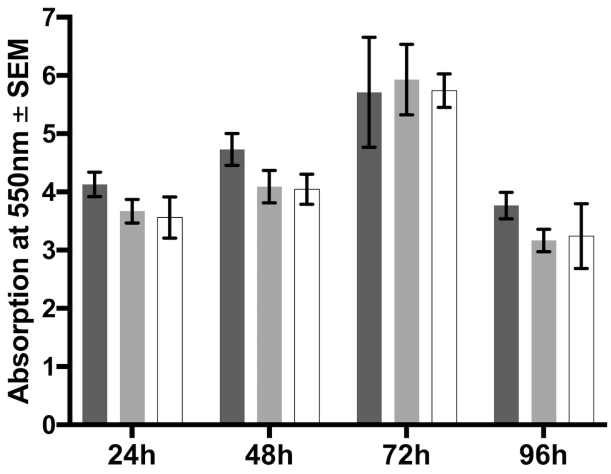

Fig. 20: Biofilm assay of OCN003. Biofilm formation of OCN003 (dark grey), OCN003 Δ*fliF*::*bla* mutant (grey), and the complemented Δ*fliF*::*bla* mutant (white). The bar graph shows the average absorbance for each strain with error bars representing the standard error of the mean (SEM). The assay was performed at 24 h, 48 h, 72 h, and 96 h post-inoculation.

wild type physiology, growth rate, and retained the ability to from a biofilm (Fig. 20). Although OCN003 Δ*fliF*::*bla* formed a flagellum (Fig. 21), it was non-functional, and the mutant could no longer swim, as confirmed by light microscopy and semi-solid agar assays (Fig. 22). When the OCN003 Δ*fliF*::*bla* strain was used to infect cMWS-afflicted *M. capitata* fragments, the mutant was incapable of inducing the switch from chronic to acute lesions (Mantel-Cox test: $n = 8$, $p < 0.01$). Introduction of a plasmid carrying a functional copy of *fliF* into the OCN003 Δ*fliF*::*bla* mutant complemented the mutation and restored its ability swim and cause the switch from cMWS to aMWS lesions at rates that were similar to that of the wild type (Fig. 23; Mantel-Cox test: $n = 8$, $p = 0.90$). The complemented OCN003 Δ*fliF*::*bla* mutant was also successfully re-isolated from all of the experimentally infected fragments. These results fulfill Koch's molecular postulates for the requirement of *fliF*, and motility by extension, for proper virulence of OCN003 as a secondary pathogen. This

indicates that wild type OCN003, not just the presence of viable OCN003 cells, is required to induce the switch from cMWS to aMWS lesions.

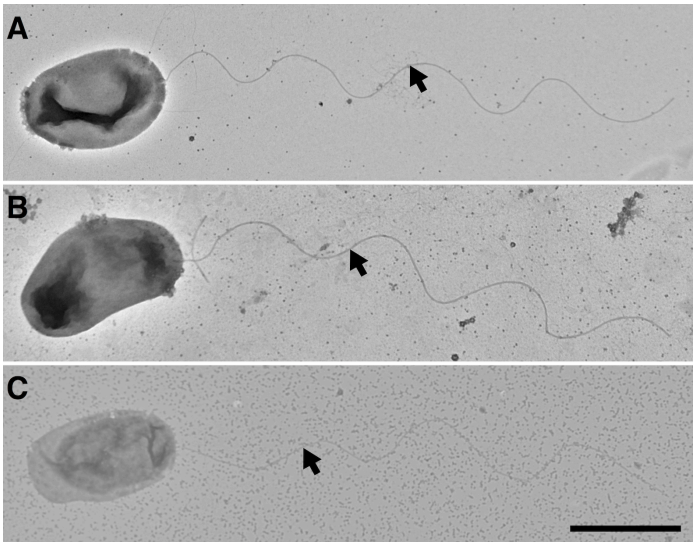

Fig. 21: Electron micrographs of contrasted preparations of OCN003 and mutants. (A) shows the wildtype, (B) the Δ*fliF*::*bla* mutant, and (C) the complemented Δ*fliF*::*bla* mutant, all exhibiting the presence of a polar flagellum (arrow). Cells used for analysis were deposited on Formvar-coated copper grids and contrasted with 1% uranyl acetate for viewing on a Hitachi HT7700 TEM at 100 kV. Images were captured with an AMT XR-41B 2k x 2k CCD camera. Scale bar represents one μm.

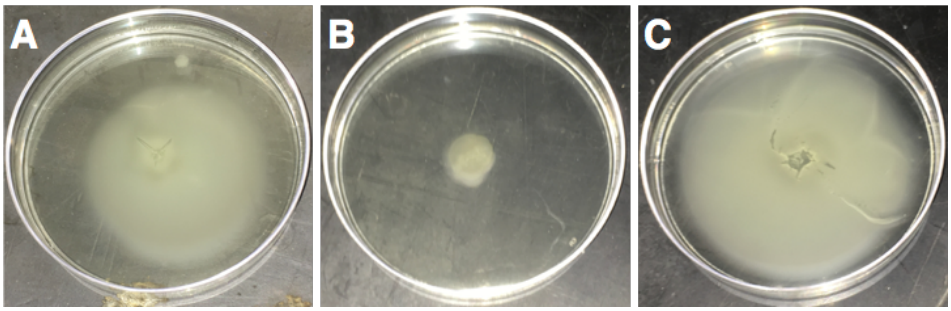

Fig. 22: Motility assay of OCN003 and mutants. Semi-solid (0.15% agar) assay confirming motility of wild type OCN003 cells (A), the lack of motility of the OCN003 Δ*fliF*::*bla* mutant cells (B), and the restored motility of the complemented Δ*fliF*::*bla* mutant cells (C).

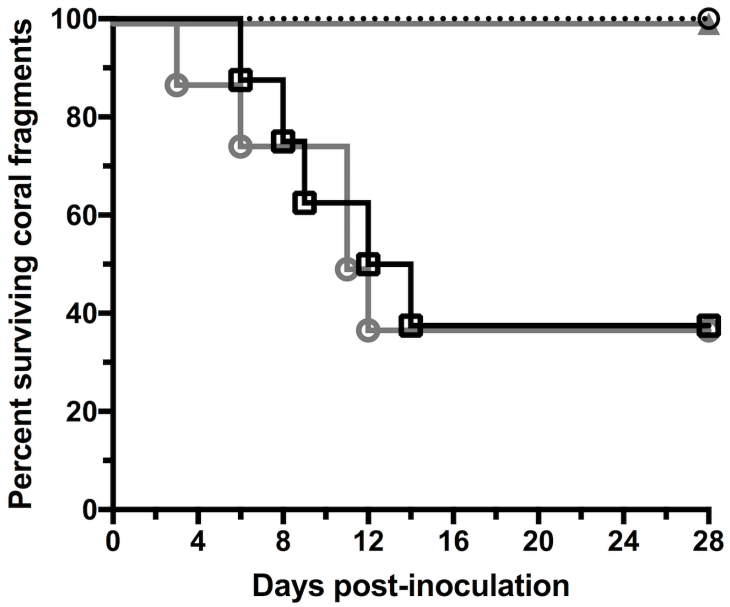

Fig. 23: Kaplan–Meier survival curve of cMWS-afflicted *M. capitata* fragments exposed to OCN003, OCN003 Δ*fliF*::*bla*, the complemented Δ*fliF*::*bla* mutant, or the control bacterium, OCN004, at 25°C. The solid line with open black squares represents fragments exposed to OCN003 (n = 8); the solid grey line with open grey circles represents fragments exposed to the complemented Δ*fliF*::*bla* mutant (n = 8); the dotted line with open black circles represents fragments exposed to OCN004 (n = 8); the solid grey line with closed grey triangles represents fragments exposed to OCN003 Δ*fliF*::*bla* (n = 8). The concentration of bacteria was 10^8 CFU/ml of seawater.

Pseudoalteromonas piratica from diseased *M. capitata* fragments collected during an aMWS outbreak in 2010.

In March of 2010 an outbreak of aMWS affected several hundred colonies of *M. capitata* (Aeby *et al.*, 2016). To determine if strains of *P. piratica* may have been involved in the outbreak, bacteria were cultured from the mucus of infected colonies and identified. A total of 265 bacterial isolates were cultured from four aMWS-infected coral fragments collected during the outbreak. These isolates were screened by PCR using previously

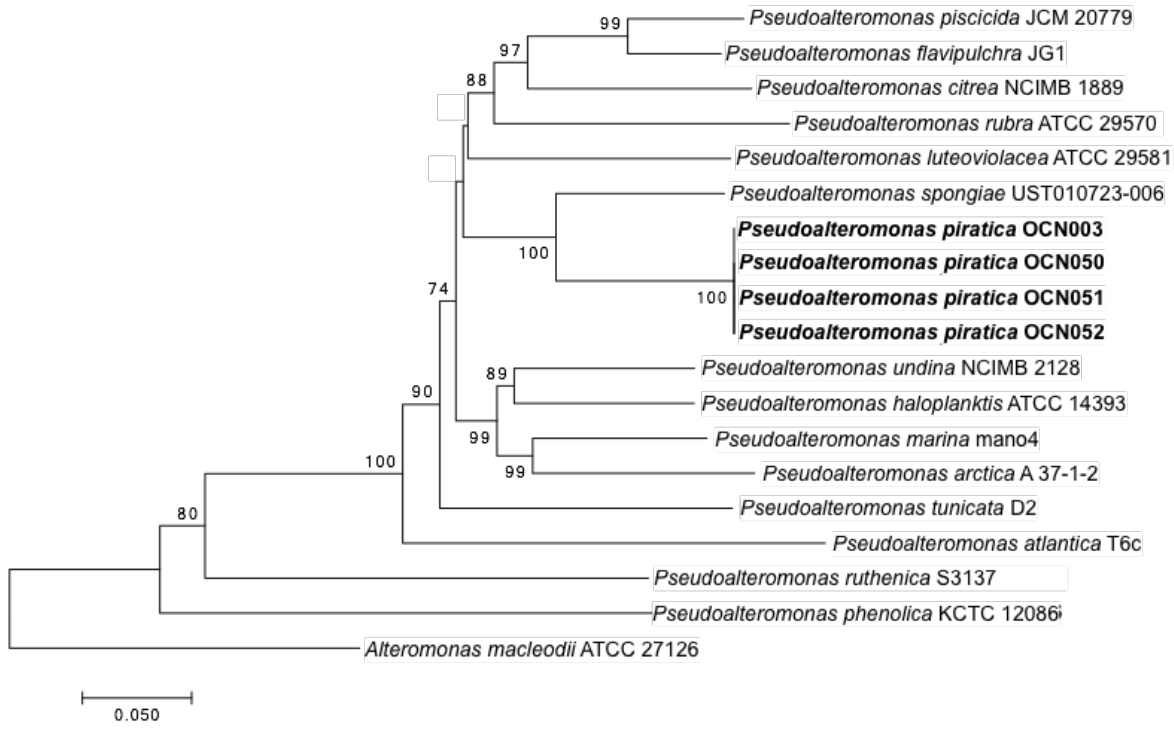

Fig. 24: Neighbor-joining dendrogram showing the estimated phylogenetic relationships between the *Pseudoalteromonas piratica* strains OCN003, OCN050, OCN051, OCN052, and related *Pseudoalteromonas* spp. based upon a modified multi-locus sequence analysis. Analysis was based upon the sequences of the housekeeping genes *recA, gapA, and ftsZ. Alteromonas macleodii* ATCC 27126 was chosen as the outgroup. The scale bar represents five nucleotide substitutions per 100 nucleotides. Bootstrap values >70% (500 replicates) are indicated at nodes.

described primers (OCN008-42310-F and OCN008-43080-R) specific to *V. coralliilyticus* strain OCN008, a pathogen of *M. capitata* (Ushijima *et al.*, 2014), or primers that selectively amplify a unique region of the OCN003 genome (003unique-F and 003unique-R). Five isolates from the 2010 outbreak grew on *Vibrio*-selective TCBS agar, but none yielded a PCR product using the OCN008-specific primers. In contrast, three of the 265 isolates yielded a PCR product when screened with the OCN003-specific primers. The 16S rRNA gene sequences from each of the three OCN003-like isolates, referred to as OCN050,

OCN051, and OCN052, were identical to the those of OCN003 (data not shown), and all three isolates clustered with OCN003 during MLSA comparison, which supports their classification as *P. piratica* strains (Fig. 24). Based on the similarity of these isolates, the remainder of the study utilized OCN050 as a representative isolate from the outbreak.

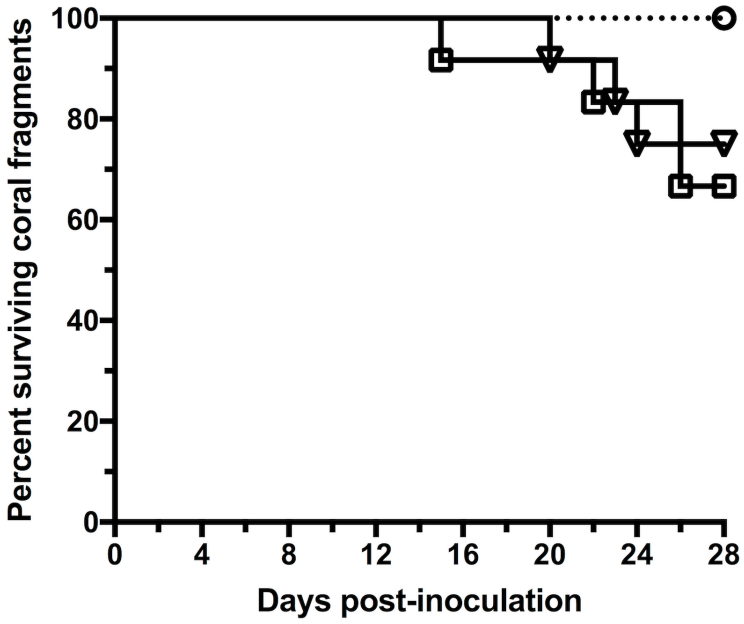

Fig. 25: Kaplan–Meier survival curve of healthy *Montipora capitata* fragments exposed to OCN003, OCN050 or the control bacterium, OCN004, at 25 ˚C. The solid line with open black squares represents fragments exposed to OCN003 (n = 12); the solid line with open black nablas represents fragments exposed to OCN050 (n = 12); the dotted line with open circles represents fragments exposed to OCN004 (n = 12). The concentration of bacteria used was 10^8 CFU/ml of seawater.

Strain OCN050 was tested for its ability to induce acute tissue loss in *M. capitata* as a primary pathogen and induce the cMWS to aMWS switch as a secondary pathogen. During infection trials, OCN050 infected fragments of *M. capitata* at levels that were similar to those of OCN003, 25% and 33%, respectively (Mantel-Cox test: n=12, p=0.71; Fig. 25). None of the negative control fragments displayed any signs of disease following exposure to FSW

or OCN004 (n = 12 per treatment). In addition, OCN050 induced a switch from cMWS to aMWS in 38% of the exposed cMWS-afflicted fragments (McNemar's test: n = 16, p = 0.04), which was not significantly different from the OCN003-mediated switch from cMWS to aMWS (Mantel-Cox test: n = 16, p = 0.50; Fig. 26). These data indicate that OCN003 and OCN050 infect *M. capitata* fragments similarly and can act as secondary pathogens to induce the switch from cMWS to aMWS lesions in laboratory conditions. Taken together, OCN003 and OCN003-like strains are etiological agents of aMWS, and may have been involved in an aMWS outbreak in 2010.

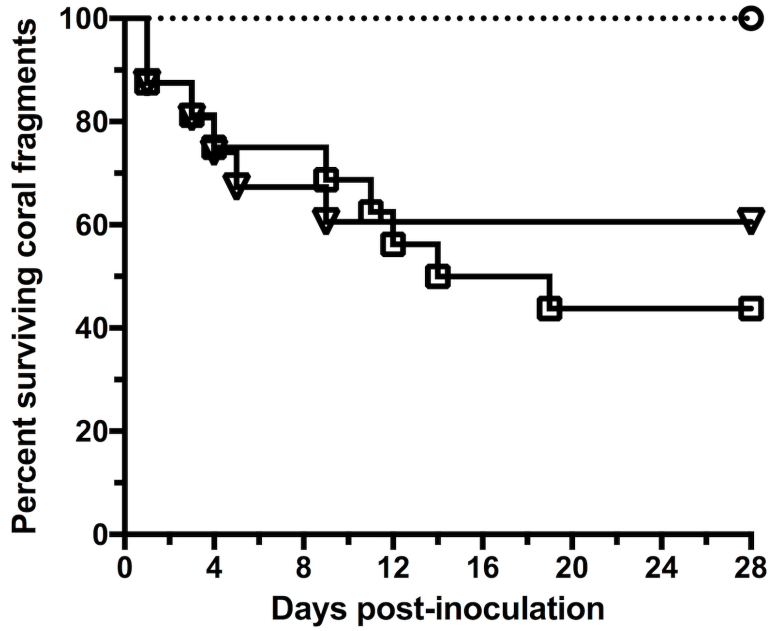

Fig. 26: Kaplan–Meier survival curve of cMWS-afflicted *M. capitata* fragments exposed to OCN003 (originally isolated from diseased *M. capitata*), OCN050 (re-isolated in diseased *M. capitata* fragment from outbreak) or the control bacterium, OCN004, at 25°C. The solid line with open black squares represents fragments exposed to OCN003 (n = 16); the solid line with open black nablas represents fragments exposed to OCN050 (n = 16); the dotted line with open circles represents fragments exposed to OCN004 (n = 16). The concentration of bacteria was 10^8 CFU/ml of seawater.

***P. piratica* strains and *V. coralliilyticus* strain OCN008 display different infection rates in secondary infections.**

Like OCN003, *Vibrio coralliilyticus* strain OCN008 has fulfilled Koch's postulates as an etiological agent of aMWS for *M. capitata* (Ushijima *et al.*, 2014). It is possible that any bacterial agent that can cause aMWS can induce a switch from cMWS to aMWS, and this is not an OCN003-specific interaction with *M. capitata*. To assess the specificity of the secondary pathogen-mediated switch from cMWS to aMWS, *M. capitata* fragments exhibiting cMWS disease signs were exposed to OCN008. When healthy coral fragments were exposed to OCN008 as a primary pathogen, 80% of the fragments developed acute tissue loss an average of two days post-inoculation (McNemar's test: n=22, $p < 0.01$). However, when aliquots of the same OCN008 cultures were used to inoculate *M. capitata* with cMWS, 46% of the fragments developed acute lesions in an average of 10 days post-inoculation (McNemar's test: n=13, p=0.04; Fig. 27). In contrast, cMWS-afflicted fragments from the same diseased colonies were more susceptible to OCN003 as a secondary pathogen, which was able to induce a switch to aMWS lesions in 62% of specimens in an average six days post-inoculation during concurrent infection trials. Analysis of the infection rates of OCN003 as a primary and secondary pathogen shows that its ability to infect *M. capitata* fragments with pre-existing cMWS lesions is significantly higher than its infection of healthy fragments (Mantel-Cox test: n_1 = 16, n_2 = 12, p = 0.03). The opposite result was found for OCN008, in which its ability to infect as a secondary pathogen was statistically lower than infection as a primary pathogen (Mantel-Cox test: n_1 = 13, n_2 = 22, $p < 0.01$). When healthy and cMWS-afflicted fragments were exposed to the control bacterium, OCN004, no fragments displayed a change in disease state. Taken together,

these results demonstrate that OCN008 infects *M. capitata* more effectively as a primary pathogen, whereas OCN003 is more effective as a secondary pathogen.

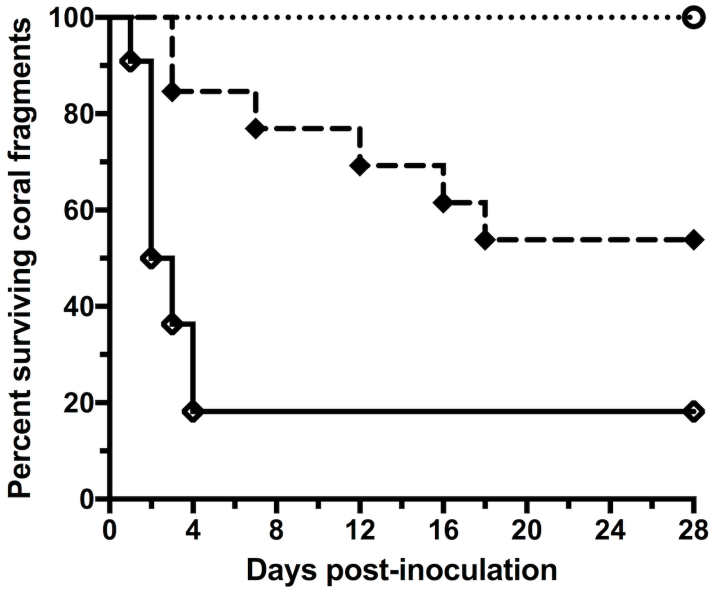

Fig. 27: Kaplan–Meier survival curve of *M. capitata* fragments exposed to OCN008 or the control bacterium, OCN004, at 25˚C. The solid line with open black rhombi represents healthy coral fragments exposed to OCN008 (n = 22); the dashed line with closed black rhombi represents cMWS-afflicted coral fragments exposed to OCN008 (n = 13); the dotted line with open circles represents healthy or cMWS-afflicted coral fragments exposed to OCN004 (n = 22 or n = 13, respectively). The concentration of bacteria was 10^8 CFU/ml of seawater.

DISCUSSION

The work presented here describes the characterization of a novel *Pseudoalteromonas* species, *P. piratica* strain OCN003, that fulfills Koch's postulates of disease causation for infection of the coral *M. capitata* as both a primary pathogen, which is capable of causing aMWS, and as a secondary pathogen, which induces the switch from

cMWS to aMWS. Strains of *P. piratica* were isolated from diseased *M. capitata* and could infect healthy *M. capitata* fragments in controlled laboratory experiments, which resulted in acute tissue loss similar to that seen on the reef (Aeby *et al.*, 2016). The type strain, OCN003, was also capable of inducing the switch from chronic to acute lesions in the laboratory, which is the first demonstration of a bacterium that can act as a secondary pathogen in coral infection. Additionally, Koch's molecular postulates were fulfilled for the involvement of motility as a virulence mechanism influencing the switch from cMWS to aMWS as a secondary pathogen. This work indicates that *P. piratica* strain OCN003 is an etiological agent of aMWS, as well as a driver of the switch from cMWS to aMWS, on *M. capitata* in Kāneʻohe Bay, Hawaiʻi.

In human disease research, studies have documented bacteria acting as secondary pathogens to bring about greater levels of lethality than either the primary or secondary pathogens alone (Witt *et al.*, 1987; Beadling and Slifka, 2004; Brundage and Shanks, 2007; Mallia *et al.*, 2012). Once the host is infected by the primary pathogen, the immune response may be modulated or weakened to facilitate infection by the secondary pathogen (Beadling and Slifka, 2004; Guzman and Vazquez, 2010; Li *et al.*, 2012). Previous work on bacterial pathogenesis in humans has shown that *Mycobacteria* can act as primary pathogens and many opportunistic strains including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and several Streptococci can infect as secondary pathogens (Brook, 2002; Yeboah-Manu *et al.*, 2013; Langbang *et al.*, 2016). In these interactions, each of the secondary pathogens can infect as a primary pathogen, but the damage done to the host is much greater when the pathogens co-infect. This scenario holds true for OCN003; it can infect as a primary pathogen causing aMWS but damages the host much more quickly as a

secondary pathogen by inducing the switch from cMWS to aMWS. However, despite the differences in infection, this work also demonstrates that two unique bacteria can elicit the same disease in a single coral species. Due in large part to the limited number of disease signs coral can display, it is easy to assume that a single pathogen or consortium is the cause of a coral disease. Based on our results, it is entirely possible that additional undiscovered pathogens may be capable of causing well-studied coral diseases.

The results presented here show that two pathogens, OCN003 and OCN008, display very different levels of virulence toward *M. capitata* as primary and secondary pathogens. OCN003 only infected 33 % of colonies roughly 22 days post-inoculation as a primary pathogen but infected 56 % of colonies about eight days post-inoculation as a secondary pathogen. This is the opposite pattern of infection observed for OCN008; 80 % of colonies were infected by OCN008 two days post-infection as a primary pathogen but only 46 % of colonies were infected about ten days post-infection as a secondary pathogen. The differences in infectivity between these strains acting as either primary or secondary pathogens suggest that some component of either the coral or the pathogen modulates virulence under these conditions.

M. capitata colonies with cMWS infections may be inherently different than uninfected colonies in that differences in the community structure of the bacterial microflora residing on the coral could modulate the infectivity of OCN003 and OCN008. It has been postulated that the microflora present in coral mucus confer some level of protection against bacterial infection either by competing for space or nutrients or by producing antimicrobial compounds to inhibit pathogen growth (Ritchie and Smith, 2004; Reshef *et al.*, 2006; Ritchie, 2006; Rosenberg *et al.*, 2007; Nissimov *et al.*, 2009; Shnit-

Orland and Kushmaro, 2009). Strains of the normal microflora have been isolated from coral and have been shown to produce various growth-inhibiting compounds including tropodithietic acid (Raina *et al.*, 2016), cembranoid (Al-Footy *et al.*, 2015), and triterpenoid (Elnaggar *et al.*, 2017) antibiotics. Additionally, recent studies have shown that the primary components of bacterial communities in coral mucus change greatly during infection (Pantos and Bythell, 2006; Sato *et al.*, 2009; Sunagawa *et al.*, 2009; Cárdenas *et al.*, 2012; Kellogg *et al.*, 2013; Pollock *et al.*, 2017). Gochfeld and Aeby (2008) demonstrated that healthy *M. capitata* colonies had significantly higher levels of antibacterial activity than those affected by MWS, and MWS-afflicted tissues had significantly higher levels of antibacterial activity than unaffected tissues, which could explain differential susceptibility to disease. Not all pathogens are sensitive to all antimicrobial compounds; OCN003 is resistant to kanamycin (100 μg) and OCN008 is resistant to both kanamycin (25 μg) and ampicillin (800 μg) (Beurmann *et al.*, *in press* and Ushijima *et al.,* 2014). It is therefore possible that OCN003 infects *M. capitata* colonies more readily as a secondary pathogen because some members of the bacterial microflora that were present in healthy mucus and inhibited OCN003 infection were absent from diseased fragments. Without the normal microflora to protect the coral, OCN003 infected more readily. The change in the microflora could also result in certain members becoming far more prominent than they are in healthy mucus. Should these bacteria inhibit the growth of OCN008, OCN008 would infect fragments of *M. capitata* with cMWS lesions less effectively than healthy fragments. Such changes in the microfloral community structure could serve to alter infection by these pathogens. While the coral provides a permissive host environment for infection, the pathogens must respond to it properly for infection to proceed.

To date, only a few organisms have individually fulfilled Koch's postulates of disease causation for coral infection: *V. coralliilyticus* strains OCN008 (Ushijima *et al.*, 2014), OCN014 (Ushijima *et al.*, 2016), P1-7 (Sussman *et al.*, 2008), and BAA-450 (Ben-Haim *et al.*, 2003); *V. owensii* strain OCN002 (Ushijima *et al.*, 2012); *Vibrio shiloi* strain AK1 (Ben-Haim *et al.*, 1999); *Serritia marcescens* strain PDR60 (Sutherland *et al.*, 2011); and *Aurantimonas coralicida* strain WP1T (Denner *et al.*, 2003). Every one of these strains belong to groups known to cause several diseases. *Vibrio* strains kill bivalves (Paillard *et al.*, 2004; Richards *et al.*, 2015), shrimp (Alvarez *et al.*, 1998), fish (Egidius *et al.*, 1986; Alvarez *et al.*, 1998), and humans (Cottingham *et al.*, 2003). *Serratia* strains infect dogs (Wilkins, 1973), birds (Saidenberg *et al.*, 2007), and humans (Acar, 1986). A strain of *Aureimonas* (previously classified as and closely related to *Aurantimonas*) infects humans (Schröttner *et al.*, 2014). Finally, the results of this study have demonstrated that strains of *Pseudoalteromonas piratica* infect the coral *M. capitata.* Members of the genus *Pseudoalteromonas* have been shown to infect many marine organisms including fish (Nelson and Ghiorse, 1999), crustaceans (Stentiford, 2008), and sponges (Choudhury *et al.*, 2015). Based on the infectivity of members of these genera, it seems that coral disease research is dominated by members of highly pathogenic genera. The commonality of these pathogenic strains isolated from diseased organisms raises the question of whether these genera comprise the most common pathogens or whether researchers knew to look for members of these genera as pathogens based on previously published work regarding their pathogenic potential. The body of work describing coral pathogens that fulfill Koch's postulates was conducted by groups in disparate areas of the world, generally using a haphazard approach to picking cultivable colonies for initial rounds of virulence testing. This attempted

randomization indicates that members of the above genera may represent the most common, easily cultivable coral pathogens. With the advent and decrease in cost of high-throughput sequencing allowing analysis of massive data sets, comparison of multiple samples from diseased coral may point toward potentially novel as-yet-uncultivable pathogens.

Multiple studies have indicated that coral pathogens can respond to many cues from their environments. For example, the coral pathogen *V. coralliilyticus* BAA-450, which infects the coral *Pocillopora damicornis*, is perhaps the best-studied example of a coral pathogen altering virulence as a response to environmental cues (Ben-Haim *et al.*, 2003). During laboratory infection trials conducted at 27 ˚C, BAA-450 induced tissue lysis in fragments of *P. damicornis*. At 24 ˚C, BAA-450 induced bleaching of the coral but infection was abrogated at 23 ˚C. A proteomic study on BAA-450 found that 136 gene products were upregulated at 27 ˚C, as compared to 23 ˚C, and they generally belonged to classes that were involved in pathogenesis in other *Vibrio* species (De O Santos *et al.*, 2011; Kimes *et al.*, 2012). Two such genes that were upregulated at 27 ˚C, *mshA* and *toxR*, were found to be required for proper virulence in OCN008 (Ushijima *et al.*, 2016). It has also been shown that BAA-450 exhibits chemotaxis toward DMSP, a metabolite produced by coral (Garren *et al.*, 2014). It is therefore possible that some of the compounds produced once *M. capitata* is infected with cMWS serve as the cue to upregulate virulence factors in OCN003. To modulate OCN008 infection, a compound may be produced by healthy coral, and absent from diseased coral, to upregulate virulence factors or, conversely, a compound is produced from diseased coral that downregulates virulence factors. Vidal-Dupiol *et al.* (2011) characterized an antimicrobial peptide, damicornin, produced by *P. damicornis* as a

response to nonpathogenic immune challenge. Expression of damicornin was repressed by the coral pathogen *V. coralliilyticus* (Vidal-Dupiol *et al.*, 2011), supporting the proposal that coral health and virulence mechanisms of the pathogens could be responsible for the altered infectivity of *M. capitata* by OCN003 and OCN008 as primary and secondary pathogens.

EXPERIMENTAL PROCEDURES

Bacterial growth conditions.

All bacterial strains used in this chapter are listed in Table 4. Marine bacteria were grown in glycerol artificial seawater (GASW) broth or on plates solidified with 1.5 % agar as previously described (Ushijima *et al.*, 2012). Bacterial cultures were incubated at 28 °C unless otherwise stated.

Escherichia coli strains were grown in LB-Miller medium and incubated at 37 °C. Antibiotics for plasmid selection in *E. coli* were used at the following concentrations unless otherwise stated: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; streptomycin, 50 μg/ml; spectinomycin, 100 μg/ml; and chloramphenicol, 30 μg/ml. Antibiotics for the selection of marine bacteria were used at the following concentrations unless otherwise stated: ampicillin, 150 μg/ml; gentamicin, 15 μg/ml; and kanamycin, 50 μg/ml. Auxotrophic *E. coli* strains were grown on media supplemented with deoxythymidine (DT) or diaminopimelate (DAP) at a final concentration of 0.3 mM each (Le Roux *et al.*, 2007). Bacterial *sacB*-mediated counterselection was achieved by supplementing GASW with 5% (w/v) sucrose (Steinmetz *et al.*, 1985).

Table 4: Strains and plasmids used in this chapter.

Strain or plasmid	Relevant characteristic(s)	Source or citation
***Pseudoalteromonas piratica* strains**		
OCN003	Wild type; isolated from the mucus of diseased *M.capitata*; Km^r	Beurmann *et al.*, 2015
OCN050	Wild type; isolated from diseased *M.capitata* during aMWS outbreak; Km^r	This study
OCN051	Wild type; isolated from diseased *M.capitata* during aMWS outbreak; Km^r	This study
OCN052	Wild type; isolated from diseased *M.capitata* during aMWS outbreak; Km^r	This study
OCN003 Δ*fliF*::*bla*	OCN003 Δ*fliF*::*bla* mutant; Ap^r, Km^r	This study
Marine bacterial strains		
OCN004	Non-pathogenic *Alteromonas* sp; negative-control bacterium	Ushijima *et al.*, 2012
OCN008	Pathogenic *Vibrio coralliilyticus* strain; Ap^r	Ushijima *et al.*, 2014
***Escherichia coli* strains**		
β3914	Δ*dapA*::(*erm-pir*); Km^r, Em^r, Tc^r	Le Roux *et al.*, 2007
π3813	Δ*thyA*::(*erm-pir*); Em^r	Le Roux *et al.*, 2007
DH5α	F- supE44 ΔlacU169 (φ80lacZΔM15) ΔargF hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Stratagene
Plasmids		
pBBR1MCS-4	Source of Ap cassette; Ap^r	Kovach *et al.*, 1994
pBluescript SK+	Cloning vector, Ap^r	Stratagene
pEVS78	Shuttle vector; Cm^r	Stabb and Ruby, 2002
pRK2013	Self-transmissible vector for conjugation; Km^r	Figurski and Helinski, 1979
pRL1383a	Replicative vector derived from RSF1010; Sp^r, Sm^r	Wolk *et al.*, 2007
pRL277	Source of *sacB*; Sp^r, Sm^r	Black *et al.*, 1993
pSW4426T	Source of Cm^r, Sp^r, and Sm^r cassettes; Cm^r, Sp^r, Sm^r	Le Roux *et al.*, 2007
pUC18-mini Tn7T-*lacZ*	Source of Gm^r cassette; Ap^r, Gm^r	Choi *et al.*, 2005
pBU187	Modified version of pSW4426T without *ccdB* or *araC*; Cm^r, Sp^r, Sm^r	This study
pSB122	Modified version of pBluescript SK+; Ap^r, Cm^r, Sp^r, Sm^r	This study
pSB123	Modified version of pBluescript SK+ with *sacB*; Ap^r, Cm^r, Sp^r, Sm^r	This study
pSB124	Modified version of pEVS78 with *fliF*-deletion construct; Cm^r	This study
pSB125	Modified version of pEVS78 with *fliF*-deletion construct and Ap^r cassette; Cm^r	This study
pSB126	Suicide vector used to create OCN003 Δ*fliF*::*bla* mutant; Ap^r, Km^r	This study
pSB127	pBluescript SK+ with *fliF*; Ap^r	This study
pSB128	pBluescript SK+ with Gm^r and *fliF*; Ap^r, Gm^r	This study
pSB129	Replicative vector derived from pRL1383a; Sp^r, Sm^r, Gm^r	This study

Abbreviation for antibiotic resistance cassettes: Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance.

Clone library creation.

Mucus samples from healthy and aMWS-infected *M. capitata* fragments were prepared and plated onto GASW agar as previously described (Ushijima *et al.*, 2012) and incubated at 25 °C for 48 h. Serial dilutions were conducted in filtered seawater (FSW), seawater filtered through a 0.22 µm pore membrane filter. Colonies were purified on GASW plates and isolates were selected based on colony morphology to maximize sample diversity. Total DNA was extracted from each isolate using the MoBio PowerSoil DNA extraction kit (MoBio Carlsbad, CA, USA) according to the manufacturer's instructions with

minor modifications. The lysis step was modified by increasing the incubation period to 20 min with two rounds of vortexing (2-3 minutes each) dispersed evenly during the 20 min period. The 16S rRNA gene was amplified using the universal primers 8F and 1513R (Aebischer *et al.*, 2006; Table 5) and the same primers were used to sequence the ~1.5 kb PCR product. Sequences were analyzed using the NCBI database and BLAST (Altschul *et al.*, 1990).

Table 5: Oligonucleotide primers used in this chapter.

Primer name	Primer sequence (5' → 3' orientation)	Source/citation
8F	AGAGTTTGA TCCTGGCTCAG	Aebischer *et al.*, 2006
1513R	GGTTACCTTTGTTACGACTT	Aebischer *et al.*, 2006
ftsZ-F	GTDATGTCWGCDATGGGHACNGCNATGATGGG	Sawabe *et al.*, 2007
ftsZ-R	TGHTTRCGTAAAAAHGCNGGDATRTCHAARTARTC	Sawabe *et al.*, 2007
gapA-F	GTNYTNTAYGGYTTTGGTCGYATYGGYCG	Sawabe *et al.*, 2007
gapA-R	ACYTGRCAGCTRTARCCAAAYTCRTTRTCRTACCASAC	Sawabe *et al.*, 2007
recA-F	CAAATTGARCGNCARTTTGGTAAAGGYTCAATYATG	Sawabe *et al.*, 2007
recA-R	RTARCTRSACCASGCRCCNGCTTTSTCAAC	Sawabe *et al.*, 2007
003unique-F	GCACACCTAGCTCATCTTCAAGCATACGTACTTG	This study
003unique-R	GGCCATCACCTAAGTTGTAATCCACTAAC	This study
pSW4426T-up-AraC-SacI	ATATATGAGCTCTTGGTAACGAATCAGACAATTGACGGCTTG	Ushijima *et al.*, 2016
pSW4426T-down-ccdB	TCTGGGGAATATAAGAGCTCCAGCTTTTGT	Ushijima *et al.*, 2016
M13-F	GTAAAACGACGGCCAGTG	Messing, 1983
M13-R	GGAAACAGCTATGACCATG	Messing, 1983
003-fliF-up-F	ATATATACTAGTATCGAGCAAGGTTACAGCGAGTGTTGTA	This study
003-fliF-OEX-R	TTGGTTCTCTTGATCAGTCATCTTCCCCGGGGGTGTCCATTGTTGTTAA	This study
003-fliF-down-OEX-F	ACAGATTTAACAACAATGGACACCCCCGGGGAAGATGACTGATCAAGAG	This study
003-fliF-down-R	ATATATACTAGTTCGCTTGAATACCGCGATCATCTACATC	This study
bla-F	ATATATCCCGGGAGCTGTTTCCTGTGTGA	This study
bla-R	ATATATCCCGGGAGCGCCAGCAGGAACGCGGGCGCGCA	This study
pEVS78-MCS-F	GCCCACCTATCAAGGTGTACTGCCTTCCAG	This study
pEVS78-MCS-R	CAAATGTAGCACCTGAAGTCAG	This study
003-fliF-F	TCTAGATAAGCGATAGTGGAGTAGGGTTGT	This study
003-fliF-R	GAGCTCGTCATCTTCGGTTAACCACGCTTTA	This study
Gm^R-F	CTCGAGAAGATCCCCTGATTCCCTTTGTCAA	This study
Gm^R-R	ATCGATTTAGGTGGCGGTACTTGGGTCGATA	This study
003-fliF-outside-F	CCATTCATACTGATGACATTTTCTTAGGC	This study
003-fliF-outside-R	CATTAATACGTCTTGCTGAACTTCACG	This study
pRL1383a-MCS-F	CGAAGTTATATTCGATGCGG	Ushijima *et al.*, 2012
pRL1383a-MCS-R	CATTATGGTGAAAGTTGGAACC	Ushijima *et al.*, 2012

Coral collection and infection trials.

Healthy and cMWS-infected fragments of *M. capitata* were collected from the fringing reef surrounding Moku o Loʻe island in south Kāneʻohe Bay, Hawaiʻi, under Special Activities Permits SAP#2013–47, SAP#2015–17, and SAP#2015-48 granted by the State of Hawaiʻi, Department of Land and Natural Resources, Division of Aquatic Resources. *M. capitata* fragments were allowed to recover from collection in a flow-through water table

at ambient temperature for at least two days prior to experimental infection trials. Infection trials employed a block design in which all coral fragments used within an experimental block were collected from the same coral colony to control for intraspecific variability in disease susceptibility.

Infection trials were conducted as previously described (Ushijima *et al.*, 2012, 2014, 2016) with minor modifications. Each fragment with a cMWS lesion was individually housed in a nine-liter tank. Tanks were filled with seven liters of FSW and the water temperature was maintained at 25 ˚C. For coral inoculation, overnight liquid cultures were diluted 1:1000 in GASW broth, and then grown to an OD_{600} (optical density measured at 600 nm) of 0.8 before being washed once and resuspended in autoclaved FSW. OCN003, the Δ*fliF*::*bla* mutant, the complemented Δ*fliF*::*bla* mutant, OCN004, OCN008, and OCN050 were inoculated to a final concentration of 10^8 CFU/ml of tank water unless otherwise stated. Dilutions were prepared as previously described in Ushijima *et al.* (2014). OCN004 was used as a negative control bacterium as previously described (Ushijima *et al.*, 2012, 2014).

Identification and phylogenetic analysis of *Pseudoalteromonas* isolates.

All oligonucleotides used in this chapter are listed in Table 5. Nucleotide sequences for the 16S rRNA gene and the genes for the multilocus sequence analysis (MLSA) for 14 *Pseudoalteromonas* species were obtained from whole genome sequences from NCBI (Table 6). For MLSA, the *ftsZ, gapA,* and *recA* genes from *P. piratica* strains OCN003, OCN050, OCN051, and OCN052 were amplified by PCR using their respective primers, then

sequenced with the same primers (Table 5), and aligned and analyzed as previously described (Beurmann *et al.*, *in press*).

Table 6: GenBank accession numbers for gene sequences and proteins used in this chapter.

Strain	16S rRNA gene	*recA*	*gapA*	*ftsZ*
Alteromonas macleodii ATCC 27126^T	CP003841	AFS36391	AFS37755	AFS38337
Pseudoalteromonas arctica A 37-1-2^T	DQ787199	ERG11012	ERG10585	ERG09017
Pseudoalteromonas atlantica T6c^T	CP000388	WP_011575995	WP_011575374	WP_011576244
Pseudoalteromonas citrea NCIMB 1889^T	X82137	ERG18422	ERG18058	ERG17458
Pseudoalteromonas flavipulchra JG1	GU325751	WP_010607203	WP_010604051	WP_010605797
Pseudoalteromonas haloplanktis ATCC 14393^T	X67024	WP_016708515	WP_016707348	WP_016709471
Pseudoalteromonas luteoviolacea ATCC 29581^T	X82144	CCQ09551	CCQ12266	CCQ10341
Pseudoalteromonas marina mano4^T	AY563031	ERG27935	ERG27540	ERG27728
Pseudoalteromonas phenolica KCTC 12086	AB607331	WP_058031021	WP_058029496	WP_058031515
Pseudoalteromonas piratica OCN003^T	KF042038	WP_038640125	WP_038641289	WP_038639463
Pseudoalteromonas piscicida JCM 20779^T	AB681918	ERG34167	ERG34147	ERG34433
Pseudoalteromonas rubra ATCC 29570^T	X82147	ERG44323	ERG44376	ERG46282
Pseudoalteromonas ruthenica S3137	AF316891	KJY97116	KJY99687	KJY98742
Pseudoalteromonas spongiae UST010723-006^T	AY769918	ERG55196	ERG52548	ERG54604
Pseudoalteromonas tunicata D2^T	Z25522	EAR29741	EAR27888	EAR28847

Re-isolation of OCN003 and OCN050 from infected coral fragments.

The re-isolation of OCN003 and OCN050 tagged with the non-self-transmissible vector pRL1383a (Wolk *et al.*, 2007) was done as previously described (Ushijima *et al.*, 2012, 2014, 2016) with some modifications. Isolates that grew on GASW plates supplemented with spectinomycin and streptomycin were screened using pRL1383a-MCS primers and the *P. piratica*-specific primers, 003unique-F and 003unique-R (Table 5). The *P. piratica*-specific primers were designed to amplify a 511 bp intergenic region in the OCN003 genome between the divergently expressed coding sequences of a putative response regulator [AIY65323], 83 % identity to chemotaxis protein CheY from *Pseudoalteromonas* sp. P1-9, and a Cys regulon transcriptional activator [AIY65324], 99 % identity to the transcriptional regulator CysB from *Pseudoalteromonas* sp. P1-9. According to BLAST analysis, the intergenic region did not share significant nucleotide similarity with any other sequence in the NCBI database. In addition, the 16S rRNA gene was amplified and

sequenced to ensure that recovered *P. piratica* isolates were all identical to the OCN003 and OCN050 stock cultures.

Plasmid Construction.

All plasmids used in this chapter are listed in Table 4. Plasmid pBU187 is a plasmid based on pSW4426T (Le Roux *et al.*, 2007). A fragment containing araC-PBAD-ccdB was removed from pSW4426T by amplifying it with PCR using the primers pSW4426T-up-AraC-SacI and pSW4426T-down-ccdB, digesting the product with *Sac*I and *Dpn*I, and then self-ligating it to create pBU187.

Plasmid pSB123 is a suicide plasmid based on pBU187 used to create genetic mutations in OCN003. The R6K *oriV*, *oriT*, and chloramphenicol resistance cassette were excised from pBU187 as a ~1.7 kb *Sac*I/*Xba*I fragment and cloned into the same sites of pBluescript SK+ (Stratagene) to create pSB122. The R6K *oriV*, *oriT*, chloramphenicol resistance cassette, and multiple cloning site were amplified from pSB122 with PCR using the primers M13-F and M13-R, and the product was cloned into the *Eco*RV sites of pRL277 (Black *et al.*, 1993) to replace the *oriV* and *oriT* and create pSB123.

Plasmid pSB126 is a suicide vector based on pSB123 used to delete all but the first and last six amino acids of the *fliF* coding region in OCN003. Regions up- and downstream of the *fli*F gene were amplified by PCR from OCN003 chromosomal DNA using the primers pairs 003-fliF-up-F and 003-fliF-OEX-R and 003-fliF-down-OEX-F and 003-fliF-down-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR (Higuchi *et al.*, 1988) and the product was cloned into the *Eco*RV site of the plasmid pEVS78 (Stabb and Ruby, 2002) to create pSB124. A fragment containing P_{bla}-*bla* was

amplified by PCR from pBBR1MCS-4 (Kovach *et al.*, 1994) with the primers bla-F and bla-R and cloned into the *Sma*I site in pSB124 to create pSB125. A fragment harboring regions up- and downstream of the *fli*F gene and P_{bla}-*bla* was amplified from pSB125 by PCR using the primers pEVS78-MCS-F and pEVS78-MCS-R. The resulting product was digested with *Spe*I and cloned into the same site in pSB123 to create the vector pSB126.

Plasmid pSB129 is a replicative plasmid based on pRL1383a used to complement the OCN003 Δ*fliF*::*bla* mutant. The *fliF* gene was amplified by PCR from OCN003 chromosomal DNA using the primers 003-fliF-F and 003-fliF-R and ligated into the *Sma*I site of pBluescriptSK+ to create pSB127. The gentamicin resistance cassette was amplified by PCR from pUC18-mini Tn7T-*lacZ* (Choi *et al.*, 2005) using the primers Gm^R-F and Gm^R-R and cloned as a *Xho*I-*Cla*I fragment into the same sites of pSB127 to create pSB128. The gentamicin resistance cassette and *fliF* gene were excised from pSB128 as a *Sac*I and *Xho*I fragment and cloned into the same sites in pRL1383a to create pSB129.

Strain creation and bacterial conjugation.

The *fliF* gene was deleted from the OCN003 genome by allelic exchange with the suicide vector pSB126. The suicide vector was introduced using tri-parental conjugation with *E. coli*. The suicide vector was maintained in *E. coli* strain β3914 (Le Roux *et al.*, 2007) and the self-transmissible vector pRK2013 (Figurski and Helinski, 1979) was maintained in strain π3813 (Le Roux *et al.*, 2007). For tri-parental conjugation, donor and recipient strains were grown overnight under antibiotic selection for plasmid maintenance and with DAP or DT as required. Overnight cultures were diluted 1:1000 in fresh culture medium, grown to an OD_{600} of 0.7, and washed three times with either GASW or LB for

Pseudoalteromonas or *E. coli* strains, respectively. The strains were then resuspended in GASW to a total volume of 30 µl, combined, and spotted onto GASW plates supplemented with DAP and DT. Conjugation spots were incubated at 28 °C for 24 h before being resuspended in GASW, washed three times with fresh GASW, and then dilutions were plated onto GASW agar supplemented with chloramphenicol, but lacking DAP or DT for counterselection against the auxotrophic *E. coli* donor strains. Chloramphenicol resistant colonies, which consisted of bacteria with the suicide vector introduced through a single recombination event, were used to inoculate GASW broth and incubated for 15 h. After incubation, cultures were washed with GASW three times, and then dilutions were plated onto GASW agar supplemented with sucrose and ampicillin to isolate double-recombinant mutants. The resulting strain, OCN003 Δ*fliF*::*bla*, was verified by PCR with the primer pair 003-fliF-outside-F and 003-fliF-outside-R, which anneal outside the region of DNA used to make the mutations.

The complementation plasmid was introduced into the OCN003 Δ*fliF*::*bla* mutant via tri-parental conjugation as described above. Colonies that grew on GASW plates supplemented with gentamicin were confirmed by PCR using the primers pRL1383a-MCS-F and pRL1383a-MCS-R and then screened for motility using light microscopy.

Microscopy.

Motility of the OCN003 Δ*fliF*::*bla* mutant, the complemented Δ*fliF*::*bla* mutant, and wild type were determined using both light microscopy and a semi-solid assay (GASW broth and 0.15 % agar) in poured plates for the differentiation of motile and non-motile cells.

Cell morphology of wild type OCN003, OCN003 ΔfliF::bla and the complementation of OCN003 ΔfliF::bla were examined using transmission electron microscopy (TEM). Overnight cultures were deposited on a Formvar-coated copper grid, contrasted with 1 % uranyl acetate, and viewed on a Hitachi HT7700 TEM at 100kV. The presence of a flagellum was examined and photographed with an AMT XR-41B 2k x 2k CCD camera.

Biofilm assays.

Biofilm formation of the wild type, mutant, and complementation mutant of OCN003 was tested using a microtiter dish biofilm formation assay as previously described (O'Toole, 2011) with minor modifications. Bacterial cells were grown overnight in GASW broth, 10 µl of overnight culture was used to inoculate each microtiter dish well (24-well dish) containing two ml of sterile GASW broth. The microtiter dish was incubated for 24 to 96 h at 28 ˚C. After incubation, the culture was aspirated and the wells were rinsed twice with deionized water. To stain the biofilm, two ml of a 0.1% crystal violet solution was added to each well and then incubated at room temperature for 15 min. The crystal violet solution was aspirated and then the plate was rinsed with water and dried overnight at room temperature. For the quantification of the biofilm, two ml of 30% acetic acid was added to each well to solubilize the crystal violet, and then the solution was transferred to a new microtiter plate and the absorbance was measured at 550 nm. The data was analyzed by one-way ANOVA, followed by Tukey's multiple comparison with no significantly different results.

CHAPTER 5: GENETIC, PHENOTYPIC, PHYLOGENETIC, AND BIOCHEMICAL DESCRIPTION OF *PSEUDOALTEROMONAS PIRATICA* SP. NOV.[2,3]

INTRODUCTION

On the basis of 16S rRNA gene nucleotide sequences, Gauthier *et al.* (1995) revised the genus *Alteromonas* to contain only *Alteromoans macleodii,* and placed the remaining species in a new genus, *Pseudoalteromonas*. While members of most cultivable bacterial genera can be isolated from both marine and terrestrial environments, there has not been a terrestrial *Pseudoalteromonas* strain isolated to date. Additionally, *Pseudoalteromonas* species are frequently found in association with eukaryotic hosts in marine environments and studies of these associations have provided insight into important mechanisms in host-microbe interactions (Lee *et al.*, 2001; Pichon *et al.*, 2007; Shnit-Orland and Kushmaro, 2009; Givan *et al.*, 2015). Many *Pseudoalteromonas* species produce biologically active metabolites with a wide range of functionalities, including antimicrobials, many of which appear to be unique to members of this genus (Holmström and Kjelleberg, 1999). The release of antimicrobial compounds may greatly benefit *Pseudoalteromonas* cells in their competition for nutrients and colonization of surfaces. Agarases, toxins, bacteriolytic substances and other enzymes that are produced by members of this genus may assist in their competition for nutrients and space while also protecting against predators grazing on colonized surfaces. Studies investigating the regulation of such metabolites have shown that the level of hydrophobicity of the substrate colonized by Pseudoalteromonads affects

[2] Beurmann *et al.* 2015. *Genome Announc.* doi: 10.1128/genomeA.01396-14

[3] Beurmann *et al.* 2017. *Int. J. Syst. Evol. Microbiol*. doi: 10.1099/ijsem.0.00199

their production of antibacterial compounds, the highest antimicrobial activity is produced during growth on hydrophilic surfaces (Ivanova *et al.*, 1998). These findings suggest that the production of compounds with antimicrobial activity can be modulated by interaction with the immediate environmental stimuli. The metabolic potential of strains from this genus has been exploited for use in aquaculture, antifouling, and the control of toxic algal blooms as valuable methods of biocontrol. Holmström *et al.* (2000) showed that *P. tunicata* cells continued to produce antifouling components while entrapped and alive in polyacrylamide gels and polyvinylalcohol gels. An antimicrobial-producing strain of *P. undina* was used as a biocontrol agent to improve the growth of farmed fish and crustaceans based on its ability to inhibit the growth of harmful bacteria and viruses (Maeda *et al.*, 1997). In addition to the wide range of growth- or colonization-inhibiting extracellular agents produced, compounds promoting the survival of other marine organisms living near *Pseudoalteromonas* species have also been discovered.

A large number of *Pseudoalteromonas* species are found in association with marine eukaryotes including mussels (Ivanova *et al.*, 1996), pufferfish (Simidu *et al.*, 1990), tunicates (Holmström *et al.*, 1998), sponges (Ivanova *et al*., 1998), and marine plants (Akagawa-Matsushita *et al.*, 1992). Some Pseudoalteromonads influence the metamorphosis of marine invertebrates including polychaetes (Huang *et al.*, 2012), corals (Tebben *et al.*, 2011), sea urchins (Huggett *et al.*, 2006), and bryozoan larvae (Dahms *et al.*, 2004), and are thought to be important for the survival and fitness of their host organism (Holmström *et al.*, 2002). The production of a biofilm and the release of signaling molecules are hypothesized to be the mechanism that triggers settlement or metamorphosis in many of the aforementioned cases. Despite the positive roles some strains play, still other

Pseudoalteromonas species have been described as etiological agents of disease in fish (Nelson and Ghiorse, 1999; Pujalte *et al.*, 2007), crustaceans (Stentiford, 2008), and sponges (Choudhury *et al.*, 2014). The fact that isolates are found globally in marine environments associated with a variety of eukaryotic hosts suggests that their adaptation and survival strategies are diverse and efficient.

RESULTS

OCN003: a motile, prosthecate and budding species of the genus *Pseudoalteromonas*

The initial characterization of this probable Pseudoalteromonad focused on phenotypic components. Colonies on marine agar are pale yellow, 1-3 mm in diameter, and convex, with a smooth surface and entire, translucent margin. Neither diffusible pigment nor gas bubbles form on marine agar. Cells are Gram-negative rods, 1.4 to 2.6 μm in length and 0.5 to 0.8 μm in width. Cells are motile by a single polar flagellum, and prosthecae are produced peritrichously. Buds can be formed at the end of prosthecae, which form on both mother and daughter cells (Fig. 28).

Biochemical analyses support strain OCN003 to be a member of the genus *Pseudoalteromonas*

For a more complete characterization of this potentially novel strain, common biochemical parameters defined for other Pseudoalteromonads were assessed. Dominant

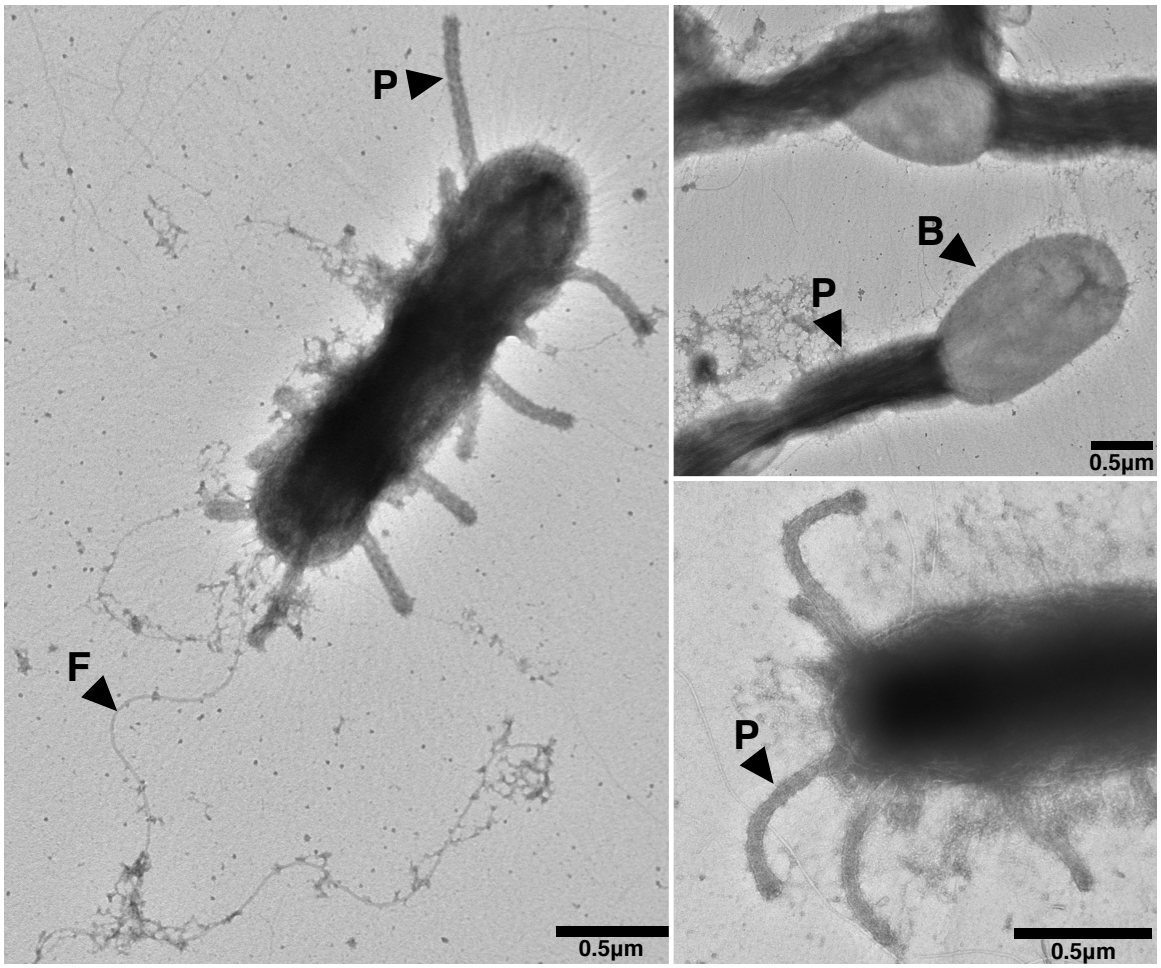

Fig. 28: Transmission electron microscopy (TEM) images of OCN003. Electron micrographs of uranyl acetate fixed cells of OCN003, showing polar flagellum (F), prosthecae (P), and buds (B).

fatty acids in OCN003 were 16:1 ω7c, 16:0, 18:1 ω7c, 17:1 ω8c, 12:0 3OH, and 17:0 (comprising 82.17 % of those named). This fatty acid profile is similar to that of *P. spongiae* and *P. ruthenica* (Table 7). OCN003 grows aerobically exclusively between 14 ˚C and 39 ˚C (but not at 13 ˚C or 40 ˚C), and only between pH 5.5 and 10 (but not at pH 5.0 or 10.5). OCN003 requires NaCl (1.0-6.0%) for growth. OCN003 is susceptible to chloramphenicol (30 µg), spectinomycin (50 µg), gentamicin (30 µg), ampicillin (100 µg), streptomycin (50 µg) and neomycin (90 µg), but is resistant to kanamycin (100 µg). The strain produces

oxidase, catalase, esterase, lipase, arginine decarboxylase, tryptophan deaminase, indole, gelatinase, and acetoin. However, lysine decarboxylase, ornithine decarboxylase, sulfide, urease, and β-galactosidase are not produced. In Biolog GN2 the strain tests positive for acetic acid, amygdalin, citrate, D-fructose, D-mannose, glycogen, glycol-L-aspartic acid, hydroxyl-L-proline, inosine, inositol, L-alanine, L-alanyl-glycil, L-aspartic acid, L-glutamic acid, L-leucine, L-proline, L-serine, L-threonine, maltose, mannitol, *N*-acetyl-D-glucosamine, propionic acid, sucrose, α-D-glucose, β-hydroxyl butyric acid, and γ-hydroxyl butyric acid (Tables 8 and 9).

Table 7: Major cellular fatty acids of OCN003^T, *Pseudoalteromonas spongiae,* and *Pseudoalteromonas ruthenica*. Numbers represent percentages of total fatty acids. Data for *P. ruthenica* and *P. spongiae* are from Ivanova et al. (2002) and Lau et al. (2005), respectively. NR=not reported.

Dominant Fatty Acid	Content (%)		
	OCN003	*P. spongiae*	*P. ruthenica*
16:1 ω7c	26.4	29.1	31.0 - 42.0
16:0	20.7	18.4	13.8 - 18.7
18:1 ω7c	15.8	6.4	4.0 - 16.0
17:1 ω8c	8.1	9.8	4.0 - 8.0
12:0 3-OH	5.7	6.9	NR
17:0	5.5	NR	NR
18:0	2.9	NR	NR
14:0	2.2	5.3	2.0 - 11.0

Phylogenetic analysis describes nearest neighbors to OCN003

While the phenotypic and biochemical evidence indicated that this strain was likely a member of the *Pseudoalteromonas* genus, sequencing of the 16S rRNA gene was required for certainty. In a BLAST alignment, the 1399 nucleotides in the 16S rRNA gene in OCN003 shared 99.4 % identity with the 16S rRNA gene sequence of *P. spongiae* UST010723-006^T,

its nearest neighbor on this basis, and only ~93-97 % with other *Pseudoalteromonas* type strains. Strain OCN003 and *P. spongiae* UST010723-006^T were adjacent in the tree (Fig. 29). A maximum likelihood tree based on the MLSA showed that OCN003 did not cluster robustly with any *Pseudoalteromonas* species type strain (Fig. 30).

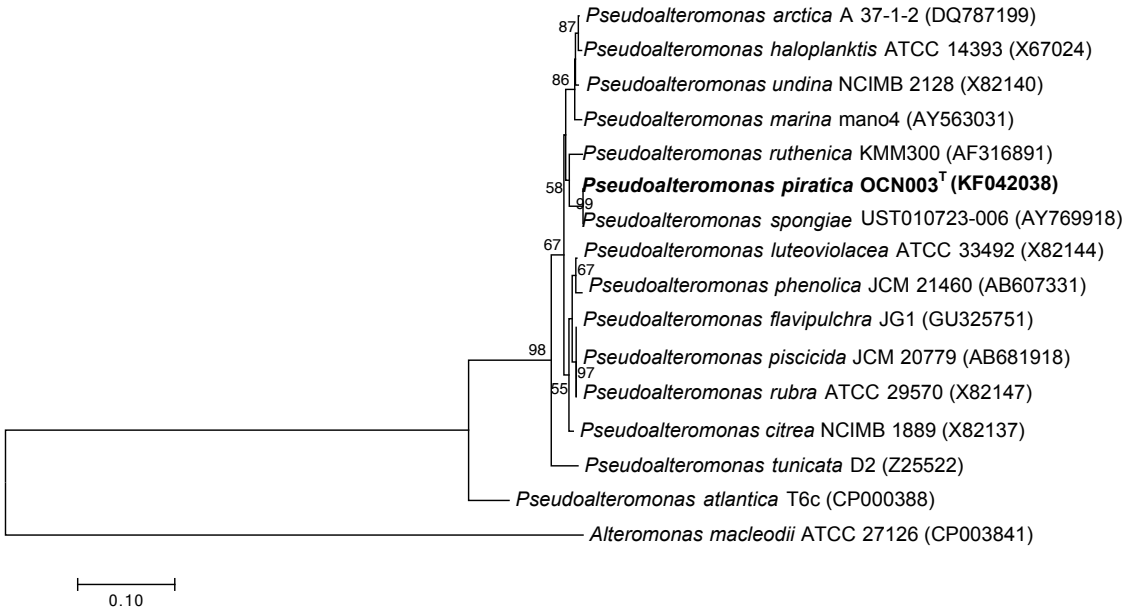

Fig. 29: Maximum likelihood dendrogram displaying the phylogenetic relationships between *Pseudoalteromonas piratica* OCN003 and related *Pseudoalteromonas* species on the basis of 16S rRNA gene sequences. *Alteromonas macleodii* ATCC 27126 was chosen as the outgroup. The scale bar represents one nucleotide substitution per 10 nucleotides. Bootstrap values >50% (500 replicates) are indicated at nodes. GenBank accession numbers for each reference strain are in parentheses.

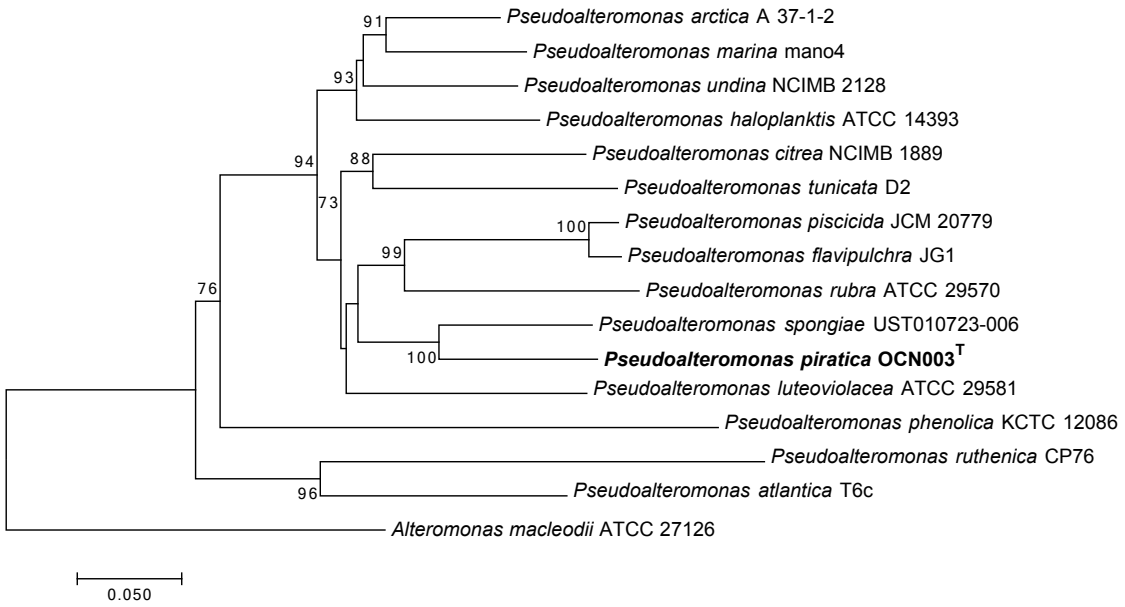

Fig. 30: Maximum likelihood dendrogram displaying the phylogenetic relationships between *Pseudoalteromonas piratica* OCN003 and related *Pseudoalteromonas* species based on a modified multi-locus sequence analysis (MLSA). Analysis was based on nucleotide sequences of the housekeeping genes *recA, gapA, and ftsZ. Alteromonas macleodii* ATCC 27126^{T} was chosen as the outgroup. Scale bar represents 5 nucleotide substitutions per 100 nucleotides. Bootstrap values >50% (500 replicates) are indicated at nodes.

DNA-DNA hybridization suggests OCN003 to be a novel species within the genus *Pseudoalteromonas*

Though the 16S sequence identified this strain as a member of the *Pseudoalteromonas* genus, additional genomic analysis was required to define it as a novel strain. Following DNA extraction and sample submission, high-throughput sequencing yielded 268,823 reads, totaling 855,362,482 bp, which were assembled into 2 high-quality contigs (Chromosome I: 3,197,498 bp, Chromosome II: 1,618,489 bp), with 40% G+C

content, a mean coverage of 138X, and 99.99205% consensus accuracy. This Whole Genome Shotgun project has been deposited at GenBank under the accession numbers CP009888 and CP009889, representing chromosome I and II, respectively. A preliminary annotation of the genome was conducted using the NCBI Prokaryotic Genome Annotation Pipeline and the Rapid Annotation using Subsystem Technology (RAST) server (Aziz *et al.*, 2008), which resulted in the identification of 4,390 genes, 94 tRNA and 22 rRNA coding sequences. The *in silico* whole genome DNA-DNA hybridization of OCN003 with its closest neighbors reflected the outcome of the phylogenetic analysis, with identities ranging from 22.8 - 25.5 %, which suggests that OCN003 is a novel species in the genus *Pseudoalteromonas* (Table 10).

DISCUSSION

The type strain, OCN003 (= CCOS 1042 = CIP 111189), was isolated from the mucus of *Montipora capitata* displaying signs of the disease acute *Montipora* White Syndrome (Aeby *et al.*, 2016) in Kāneʻohe Bay, Oʻahu, Hawaiʻi. OCN003 differs from type strains of existing *Pseudoalteromonas* species by the presence of prosthecae and buds (Fig. 28) (Gauthier *et al.*, 1995). OCN003 can also be distinguished from its four closest neighbors (*P. spongiae, P. ruthenica, P. phenolica,* and *P. luteviolacea*) on the basis of 16S rRNA gene nucleotide sequence, by 13–29 physiological traits, particularly arginine decarboxylase production, mannitol utilization (Table 8). Molecular evidence, together with phenotypic characteristics, support OCN003^{T} representing the type strain of a novel species in the genus *Pseudoalteromonas*. The species name *piratica* was chosen referring to the resemblance of OCN003 cells with 'buds' and historic representations of marauding pirates.

MATERIALS AND METHODS

Phenotypic characterization of strain OCN003

Mucus from a tissue loss disease lesion on a *M. capitata* colony in Kāneʻohe Bay, Oʻahu, was spread on glycerol artificial seawater agar (GASW) and incubated at 28˚C for 24 h. Purity was assessed by microscopic observation (e.g., of Gram-stained cells), and consistency of colony morphology on MA. Unless otherwise stated, all characteristics described herein are based on cultures grown on MA or in marine broth (MB) for 48 h at 30˚C. Cell morphology was examined by transmission electron microscopy (TEM). An overnight culture of OCN003 was deposited on a Formvar-coated copper grid, fixed with 1% uranyl acetate, and viewed on a Hitachi HT7700 TEM at 100kV. Motility was determined by light microscopy of a hanging drop preparation and observation of growth in motility medium (Smibert and Krieg, 1994).

Biochemical characterization of strain OCN003

Dominant fatty acids were determined according to MIDI Sherlock® Microbial Identification System v. 6.2, by Microbial ID, Inc. (Sasser, 1990). Whether OCN003 can grow anaerobically was examined in the GasPak™ 100 System, according to the manufacturer's instructions. The concentration of sodium chloride required for growth was tested using a previously described protocol in a medium containing 5.0 g $MgCl_2$, 2.0 g $MgSO_4$, 0.5 g $CaCl_2$, 1.0 g KCl, 5.0 g peptone (per liter) and various NaCl concentrations (0 - 80 g/L) and a pH adjusted to 7.5 with KOH (Smibert and Krieg, 1994). The pH range for growth was determined in MB using the following buffers: pH 3.0–4.0, glycine/HCl; pH 4.0–8.0, citrate/Na_2HPO4; pH 6.0–8.0, phosphate buffer; pH 9.0–11, glycine/NaOH (McCauley *et al.*,

2015). The pH was adjusted prior to sterilization, verified after sterilization prior to inoculation, and re-verified at the end of the incubation. Susceptibility to antibiotics was tested in a liquid medium inoculated with 10^4 CFU/ml of OCN003. If the optical density at 600 nm (OD_{600}) was less than 0.05 after 24 h of incubation, OCN003 was considered susceptible to the antibiotic. Oxidase and catalase activities were determined using commercially available reagent stains and droppers (BD Difco). Enzymatic activity and single carbon source utilization were determined in API 20E (bioMérieux) and GN2 MicroPlate (Biolog) kits. API 20E strips were scored after 4 h incubation at 30 ˚C, and the GN2 MicroPlates were scored after 24 and 48 h at 30 ˚C. All other tests were performed according to the manufacturers' recommendations, with the exception that the final NaCl concentration of the suspension medium for the API 20E and Biolog GN2 kits was adjusted to 2% (w/v) NaCl.

DNA extraction and genome sequencing

Genomic DNA from an axenic culture of OCN003 was isolated using a phenol-chloroform extraction method and sequenced using the PacBio RS II system at the University of California, Irvine (UCI) Genomic High-Throughput Facility. The libraries were constructed using the PacBio SMRTbell template prep kit 1.0, annealing of the sequencing primer was done according to PacBio guidelines, and sequencing was performed using the DNA/polymerase binding kit P5 and the PacBio DNA sequencing reagent 3.0. The sequencing reads were assembled using the PacBio SMRT Analysis software version 2.3.0. A preliminary annotation of the genome was conducted using the NCBI Prokaryotic Genome Annotation Pipeline and the Rapid Annotations using Subsystems Technology

(RAST) server.

Phylogenetic characterization of strain OCN003

Genomic DNA was extracted from OCN003 with the MoBio PowerSoil® DNA isolation kit (MoBio Laboratories Inc.) and used in a polymerase chain reaction with primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Aebischer *et al.*, 2006) to amplify a fragment of the 16S rRNA gene. The PCR product was purified with the QIAquick PCR Purification Kit (QIAGEN Inc.), and sequenced by Sanger sequencing in the Advanced Studies in Genomics, Proteomics and Bioinformatics facility at the University of Hawai'i at Mānoa using the same primers. This nucleotide sequence and those in 14 other species in the genus were aligned in MEGA v7.0.20 (Kumar *et al.*, 2016), and a maximum likelihood phylogenetic tree was generated. A modified multi-locus sequence analysis (MLSA) based on a method to distinguish *Vibrios* was used to further investigate the relationship between OCN003 and other members of the *Pseudoalteromonas* (Sawabe *et al.*, 2007). Nucleotide sequences from the protein-coding housekeeping genes *recA*, *gapA*, and *ftsZ* were retrieved from the complete genome sequence of OCN003 [CP009888, CP009889] (Beurmann *et al.*, 2015), and from 31 other *Pseudoalteromonas* species (Table 11). Individual sequences were retrieved from whole genomes annotated in RAST (Aziz *et al.*, 2008), concatenated and aligned in MEGA using the ClustalW program (Kumar *et al.*, 2016), and confirmed to be homologous to those of strain OCN003. *In silico* whole sequence analysis with its closest neighbor was conducted to confirm the MLSA approach.

Table 8: Differentiation of OCN003^T from other type strains of *Pseudoalteromonas* species. Data for *P. spongiae, P. ruthenica, P. phenolica,* and *P. luteoviolacea* are from Lau *et al.* (2005), Ivanova *et al.* (2002), Isnansetyo and Kamei (2003), and Gauthier (1982), respectively. +, positive; -, negative; ND, not described. All strains are oxidase- and gelatinase-positive. All strains utilize α-D-glucose, but not glycerol, L-arabinose, rhamnose, or sorbitol.

Characteristic	**OCN003^T**	***P. spongiae***	***P. ruthenica***	***P. phenolica***	***P. luteoviolacea***
Color	Pale yellow	Pale orange	Pale orange	Brown	Purple/yellow
Gas bubble formation	-	+	ND	ND	ND
Flagella	+	-	+	+	+
Motility	+	-	+	+	+
Prosthecae	+	-	ND	ND	-
Budding	+	-	ND	ND	-
NaCl (%) range for growth	1.0 - 6.0	2.0 - 6.0	1.0 - 9.0	1.0 - 5.0	3.0 - 6.0
Temp. (˚C) range for growth	14.0 - 39.0	8.0 - 44.0	10.0 - 35.0	18.0 - 37.0	10.0 - 30.0
pH	5.5 - 10.0	5.0 - 10.0	6.0 - 10.0	6.5 - 9.5	> 6.0
G+C mol%	40.0	40.6	48.4 - 48.9	39.9 - 40.6	40.9 - 42.2
Production of:					
Catalase	+	+	+	-	-
Lipase	+	-	+	+	+
Arginine decarboxylase	+	-	-	-	-
Tryptophan deaminase	+	-	ND	ND	-
Indole	+	-	ND	ND	-
Gelatinase	+	+	+	+	+
Acetoin	+	-	ND	ND	ND
Biolog GN2:					
Amygdalin	+	-	ND	ND	ND
Citrate	+	-	-	-	ND

D-Cellobiose	-	-	+	ND	-
D-Fructose	+	+	-	ND	-
D-Mannose	+	+	-	+	-
D-Trehalose	-	-	-	+	+
Glycogen	+	-	ND	ND	ND
Inositol	+	+	-	-	-
L-Leucine	+	ND	ND	-	-
L-Threonine	+	ND	ND	-	+
Maltose	+	+	-	+	+
Mannitol	+	-	-	-	-
N-Acetyl-D-glucosamine	+	+	-	+	ND
Sucrose	+	-	+	+	-
Susceptible to:					
Kanamycin	- (100 µg)	- (100 µg)	+/-	ND	-
Chloramphenicol	+ (30 µg)	+ (0.1 µg)	ND	ND	+
Ampicillin	+ (100 µg)	+ (0.1 µg)	ND	ND	ND
Streptomycin	+ (50 µg)	- (100 µg)	- (10 µg)	ND	-

Table 9: Metabolic profile of OCN003^T. Substrate metabolism was confirmed using respiratory activity in the presence of sole carbon sources through reduction of tetrazolium chloride dye in Biolog GN2. A "+" indicates the substrate was metabolized, and "-" indicates no reaction. Plates were read after incubation at 30 ˚C for 24h and 48 h.

Carbohydrate	Acid production	Carbohydrate	Acid production
α-Cyclodextrin	-	p-Hydroxy Phenylacetic Acid	-
Dextrin	-	Itaconic Acid	-
Glycogen	+	α-Keto Butyric Acid	-
Tween 40	+	α-Keto Glutaric Acid	-
Tween 80	+	α-Keto Valeric Acid	-
N-Acetyl-D- galactosamine	-	D,L-Lactic Acid	-
N-Acetyl-D- glucosamine	+	Malonic Acid	-
Adonitol	-	Propionic Acid	+
L-Arabinose	-	Quinic Acid	-
D-Arabitol	-	D-Saccharic Acid	-
D-Cellobiose	-	Sebacic Acid	-
i-Erhthritol	-	Succinic Acid	-
D-Fructose	+	Bromo Succinic Acid	-
L-Fructose	-	Succinamic Acid	-
L-Fucose	-	Glucuronamide	-
D-Galactose	-	L-Alaninamide	-
Gentiobiose	-	D-Alanine	-
α-D-Glucose	+	L-Alanine	+
m-Inositol	-	L-Alanyl-glycine	+
α-D-Lactose	-	L-Asparagine	-
Lactulose	-	L-Aspartic Acid	+
Maltose	+	L-Glutamic Acid	+
D-Mannitol	-	Glycyl-L-Aspartic Acid	+

D-Mannose	+	Glycyl-L-Glutamic Acid	+
D-Melibiose	-	L-Histidine	-
β-Methyl- D-Glucoside	-	Hydroxy-L-Proline	+
D-Psicose	-	L-Leucine	+
D-Raffinose	-	L-Ornithine	-
L-Rhmanose	-	L-Phenylalanine	-
D-Sorbitol	-	L-Proline	+
Sucrose	+	L-Pyroglutamic Acid	-
D-Trehalose	-	D-Serine	-
Turanose	-	L-Serine	+
Xylitol	-	L-Threonine	+
Methyl Pyruvate	-	D,L-Carnitine	-
Mono-Methyl-Succinate	-	γ-Amino Butyric Acid	-
Acetic Acid	+	Urocanic Acid	-
Cis-Aconitic Acid	-	Inosine	+
Citric Acid	-	Uridine	-
Formic Acid	-	Thymidine	-
D-Galactonic-Acid Lactone	-	Phenyethylamine	-
D-Galacturonic Acid	-	Putrescine	-
D-Gluconic Acid	-	2-Aminoethanol	-
D-Glucosaminic Acid	-	2,3-Butanediol	-
D-Glucuronic Acid	-	Glycerol	-
α-Hydroxy Butyric Acid	-	D,L-α-Glycerol Phosphate	-
β-Hydroxy Butyric Acid	+	Glucose-1-Phosphate	-
γ-Hydroxy Butyric Acid	+	Glucose-6-Phosphate	-

Table 10: Characteristics of sequenced *Pseudoalteromonas* genomes. Accession numbers: *Pseudoalteromonas* sp. OCN003 (CP009888, CP009889), *P. spongiae* UST010723-006 (AHCE02000000), *P. ruthenica* CP76 (AOPM00000000), *P. phenolica* KCTC 12086 (CP013187, CP013188), and *P. luteoviolacea* B = ATCC 29581

Organism	G+C content (mol%)	DDH estimate with OCN003 (%)
P. piratica OCN003	40.0	100.0
P. spongiae UST010723-006	40.6	25.5
P. ruthenica CP76	47.6	22.8
P. phenolica KCTC 12086	40.6	25.3
P. luteoviolacea B = ATCC 29581	41.9	25.0

Table 11: GenBank accession numbers for *Alteromonas* and *Pseudoalteromonas* gene and protein-coding sequences used in this chapter.

	16S rRNA gene	RecA	GapA	FtsZ	Whole Genome
A. macleodii ATCC 27126^T	CP003841	AFS36391	AFS37755	AFS38337	ABQB00000000
P. arctica A 37-1-2^T	DQ787199	ERG11012	ERG10585	ERG09017	AHBY00000000
P. atlantica T6c^T	CP000388	WP_011575995	WP_011575374	WP_011576244	AAKP00000000
P. citrea NCIMB 1889^T	X82137	ERG18422	ERG18058	ERG17458	AHBZ00000000
P. flavipulchra JG1	GU325751	WP_010607203	WP_010604051	WP_010605797	AJMP00000000
P. haloplanktis ATCC 14393^T	X67024	WP_016708515	WP_016707348	WP_016709471	AHCA00000000
P. luteoviolacea ATCC 29581^T	X82144	CCQ09551	CCQ12266	CCQ10341	CAPN00000000
P. marina mano4^T	AY563031	ERG27935	ERG27540	ERG27728	AHCB00000000
P. phenolica KCTC 12086	AB607331	WP_058031021	WP_058029496	WP_058031515	CP013187, CP013188
P. piratica OCN003^T	KF042038	WP_038640125	WP_038641289	WP_038639463	CP009888, CP009889
P. piscicida JCM 20779^T	AB681918	ERG34167	ERG34147	ERG34433	AHCC00000000
P. rubra ATCC 29570^T	X82147	ERG44323	ERG44376	ERG46282	AHCD00000000
P. ruthenica S3137	AF316891	KJY97116	KJY99687	KJY98742	AOPM00000000
P. spongiae UST010723-006^T	AY769918	ERG55196	ERG52548	ERG54604	AHCE00000000
P. tunicata D2^T	Z25522	EAR29741	EAR27888	EAR28847	AAOH00000000
P. undina NCIMB 2128^T	X82140	ERG59841	ERG62213	ERG61578	AHCF00000000

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